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THE FUNCTION OF SMC5/6 IN *DROSOPHILA* DEVELOPMENT AND AT THE HETEROCHROMATIN-LIKE LOCUS *HMR* IN BUDDING YEAST

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The function of Smc5/6 in *Drosophila* development and at the heterochromatin-like locus *HMR* in budding yeast

THESIS FOR LICENTIATE DEGREE

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ABSTRACT

The structural maintenance of chromosome 5/6 (Smc5/6) complex is one out of many factors in the cell that maintain genome stability. Smc5/6 has been implicated in processes such as DNA double-strand break (DSB) repair, replication progression, and chromosome segregation. We have examined Smc5/6 in both fruit flies (*Drosophila melanogaster*) and budding yeast (*Saccharomyces cerevisiae*) during unchallenged conditions in order to further explore its function(s) in genome stability maintenance.

In paper I we investigated early development in *Drosophila*, and found that Smc5/6 is maternally contributed. In absence of the complex, genome instability is observed during both oogenesis and embryogenesis. Oocytes lacking either Smc5 or Smc6 exhibited unrepaired DSBs, which interfered with early onset of embryogenesis. In addition, early developing embryos lacking Smc5/6 accumulated damaged nuclei. These could, however, be eliminated through a process called nuclear fallout, which allows the flies to tolerate genomic instability caused by the absence of Smc5/6 during early embryo development.

In paper II, we addressed the functional relevance of Smc6 binding to the transcriptionally repressed (also known as silenced) mating-type locus *HMR* in budding yeast. We investigated silencing of a crippled *HMR* locus *HMRae* in an *smc6-56* mutant and found that the cells contained chromosomal rearrangements in the *HMR* region. These were dependent on Rad52, a key player for homologous recombination. Moreover, *HMRae* cells lacking Topoisomerase 1 (top1) exhibited a similar recombination-dependent phenotype that was epistatic to *smc6-56*. Taken together with earlier observations, this suggests that resolution of replication-induced superhelical tension by Smc5/6 and Top1 is important for the genomic integrity of *HMR*.

Both papers demonstrate the occurrence of genomic instability in the absence of fully functional Smc5/6 complex during unchallenged conditions, and reveal an important role of the Smc5/6 complex during development in higher eukaryotes.

LIST OF SCIENTIFIC PAPERS

The following article and manuscript form the basis of this thesis. Referral to them along the text will be made according to their roman numerals.

I. **Tran M**, Tsarouhas V, Kegel A. Early development of Drosophila embryos requires Smc5/6 function during oogenesis. *Biol Open*. 2016;5(7):928-41

II. **Tran M,** Sjögren S, Kegel A. Smc6 protects the heterochromatin-like locus HMR in budding yeast from aberrant recombination events. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

Abf	ARS-binding factor
Ac	Acetyl group
AE	Axial element
ALT	Alternative lengthening of telomeres
ATR	Ataxia-telangiectasia and Rad3-related
ATM	Ataxia-telangiectasia mutated
BIR	Break-induced replication
BRCA	Breast cancer
C(2)M	Crossover suppressor on 2 of manheim
C(3)G	Crossover suppressor on 3 of gowen
Cdc	Cell division cycle
Cdt	Cyclin dependent transcript
CE	Central element
ChIP	Chromatin immunoprecipitation
CMG	Cdt1- MCM2-7 - GINS
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dHJ	Double holliday junction
Dpb	DNA polymerase B (II)
DSB	Double-strand break
E	Essential (silencer element)
EID	E1A-like inhibitor of differentiation
G1/2	Gap 1 / 2 (phase)
GINS	Go-ichi-ni-san (5-1-2-3 in Japanese) refer to the numbers of the 4 subunits in the complex; Sld5, Psf1, Psf2, and Psf3
HA	Hemoagglutinin
HJ	Holliday junction
HMR	Hidden MAT Right
HML	Hidden MAT Left
HR	Homologous recombination
H3k9me	Methylated lysine 9 of histone H3
I	Important (silencer element)
Mad	Mitotic arrest deficient
MAGE	Melanoma antigen
Mec	Mitosis entry checkpoint
Mei	Meiotic
Mcm	Minichromosome maintenance

MMS	Methyl methanesulfonate
Mre	Meiotic recombination
MRX	Mre11- Rad50 -Xrs2
Mus	MMS and ultraviolet sensitive
NHEJ	Non-homologous end joining
Nse	Non-Smc element
ORC	Origin Recognition Complex
Ord	Orientation disruptor
Ori	Origin of replication
PBS	Phosphate buffered saline
Pch	Pachytene checkpoint
PCR	Polymerase Chain Reaction
Pre-RC	Pre-replicative complex
Psf	Partner of Sld5
Rad	Radiation sensitive
Rap	Repressor activator protein
rDNA	Ribosomal DNA
RING	Really interesting new gene
Rmi	RecQ mediated genome instability
RPA	Replication protein A
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
qPCR	Quantitative PCR
S	Synthesis (phase)
SAC	Spindle assembly checkpoint
Sae	Sporulation in the absence of spo eleven
SC	Synaptonemal complex
Sds	Sodium dodecyl sulphate
SDSA	Synthesis-dependent strand annealing
Sgs	Slow growth suppressor
Sir	Silencing information regulator
Sld	Synthetic lethal with Dpb11-1
Smc	Structural maintenance of chromosomes
Spo	Sporulation
Tel	Telomere maintenance
TF	Transverse filament
Top	Topoisomerase
Xrs	X-ray sensitive

INTRODUCTION

It is important that the hereditary material, the DNA, is passed on faithfully during cell divisions. For successful cell division, DNA needs to be copied in a process called DNA replication as well as distributed equally between the mother- and daughter –cell in a process called chromosome segregation. This can be challenging as DNA is readily subjected to damage during either of these processes. Thus, a multitude of proteins are involved in accurate DNA replication, DNA repair, and chromosome segregation. Smc5/6 has an implicated role in all three areas, whereby drastic consequences are readily demonstrated by the few clinical case studies reported of patients with dysfunctional Smc5/6. These patients present developmental disorders and pleiotropic phenotypes ranging from immunodeficiency to primordial dwarfism (1, 2). Moreover, mutations in Smc5 were recently shown to be associated with brain metastasis in cancer (3). Altogether, a deeper understanding of how Smc5/6 contribute to genome stability is needed to possibly improve treatment of these diseases and disorders. In paper I we aimed to gain further insight into the function of Smc5/6 during early development by studying the fruit fly (*Drosophila melanogaster*). In paper II we investigated the binding of Smc6 to the silenced mating-type locus *HMR* in budding yeast (*Saccharomyces cerevisiae*) in order to elucidate a possible novel function at heterochromatin-like regions.

1 DNA REPLICATION, MITOSIS, AND MEIOSIS

The life of a cell can be viewed as a cycle consisting of four phases: gap phase 1 (G1), synthesis (S), gap phase 2 (G2), and cell division also known as mitosis. Cells in G1 grow and synthesize proteins in preparation for DNA replication. During S-phase the DNA is replicated and in G2 the genetic material is checked and repaired if needed. Alongside these processes, external and internal conditions are evaluated to see if it is appropriate to commit to cell division. Examples of such conditions could be nutrient levels, temperature, or other factors that can perturb the cell division process. During mitosis the replicated chromosomes segregate from each other and migrate to opposite poles of the nucleus, which then divides into two genetically identical nuclei. This ensures that each daughter cell receives a copy of every chromosome.

1.1 DNA REPLICATION

Before a cell divides, the DNA has to be duplicated to ensure proper chromosome separation into two nuclei. Our DNA consist of approximately three billion base pairs arranged in a specific order, which is copied in a semi-conservative manner each time DNA replication takes place. The DNA contains four different deoxynucleotide phosphates that are hydrogen bonded in specific (base) pairs that are as follows: adenosine - thymine and guanosine - cytosine. The base pairs are arranged in a double-helix, whereby each strand is anti-parallel to the other (4).

DNA replication starts from sites known as origins of replication (Oris) and proceed bi-directionally (Fig. 2). Prokaryotes have only one Ori, whereas eukaryotes have many. Notably, eukaryotes temporally regulate origin activation, which ensures genome duplication at defined times during S-phase. The temporal order of DNA replication is achieved through early- and late- firing origins (Fig. 1), whereby the firing of dormant origins is tightly checkpoint-regulated and activated when the replication fork progress is inhibited. As described in paper I a large number of Oris are activated during early embryo development in *D. melanogaster* in order to expedite DNA replication during rapid nuclear division cycles.

In order for DNA replication to start in S-phase and not at any other cell cycle phase, origins need to be authorized before the DNA replication can initiate (5, 6). The authorization is also called licensing and occurs during G1 (7). The Oris are fully licensed when the pre-replicative complexes (pre-RCs) have established on them. The proteins that are essential to initiate the assembly of pre-RCs are the following; ORC (**o**rigin **r**ecognition **c**omplex), Cdc6/18 (**c**ell **d**ivision **c**ycle 6/18) and Cdt1 (**c**dc10-**d**ependent **t**ranscript 1) (8, 9). The licensing proteins in turn recruit the replicative helicase complex, MCM2-7 (**m**ini**c**hromosome **m**aintenance) and the licensing is completed when MCM2-7 is loaded (10).

The pre-RC formation must be conducted and finalized in G1 such that initiation of DNA replication occurs only once and during S-phase. Re-licensing and re-replication is prevented

through the dissociation of pre-RC components after licensing has been completed and also by high cyclin-dependent kinase activity during S-phase (10-12). The latter promotes degradation or removal of the pre-RC components. If regulation of re-licensing fails, some regions would be over-replicated, which would give rise to missegregation during cell division (10, 13, 14).

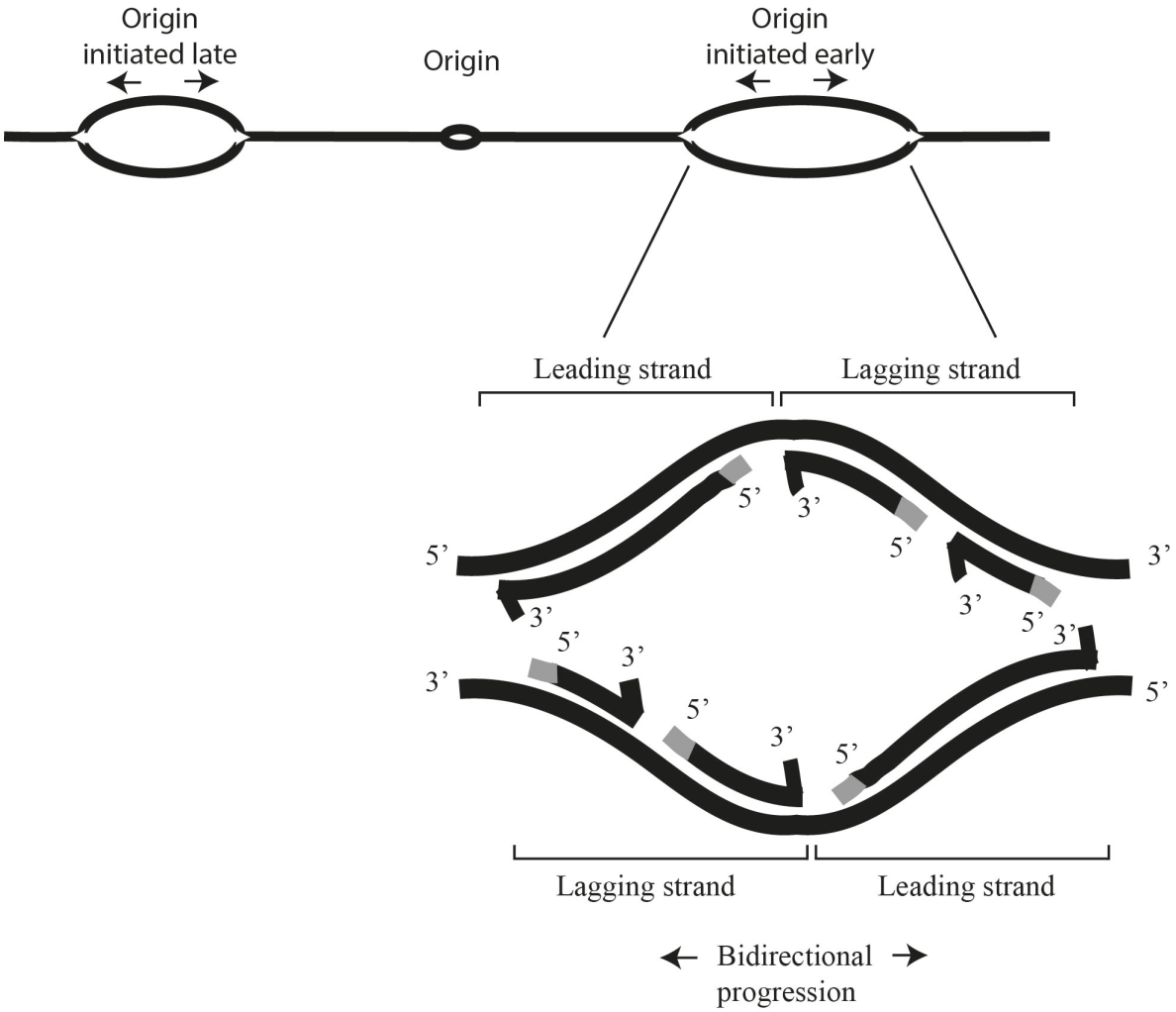

Figure 1. Illustration of origins and replication progression upon origin activation. Origins of replication (Oris) can be subdivided into late or early firing origins depending on when DNA replication is initiated during S-phase. In the figure above, the origin furthest to the right initiated early. Conversely, the origin furthest to the left initiated late, whereas the origin in between has not even initiated yet. The edge or end of the bubble have the appearance of a fork and is hence termed the replication fork.

Once an Ori has been licensed the replication machinery, known as the replisome, is assembled. It has been estimated to contain about 150 proteins that are organized around a few centers (15). Among the replisome proteins are the MCM2-7 complex, GINS (**g**o-**i**chi-**n**i-**s**an), and Cdc45. Together these three proteins of the replisome form a replicative CMG helicase, which forms a center for the assembling of the replisome proteins organize around. Upon activation of CMG, MCM2-7 unwinds and separates the DNA into two strands (16). While one strand is used as a template, deoxynucleotide triphosphates are incorporated as

deoxynucleotide phosphates into the free 5' end of the nascent strand such that it extends in a 5' to 3' direction by DNA polymerases (further reviewed in (17). Due to the anti-parallel arrangement of the DNA double helix one nascent strand can be synthesized as one continuous segment, whereas the other is synthesized in segments. The former is known as the leading strand and the latter the lagging strand. The lagging strand is synthesized in several short sections (Fig 2) called Okazaki fragments as a means to overcome the inability of polymerases to synthesize strands in 3' to 5' direction.

Unlike replication initiation, termination has not been linked to a specific DNA sequence and is less characterized. Replication termination is thought to occur via the convergence of two replication forks and involves completion of DNA synthesis, decatenation of daughter strands, and replisome disassembly (further reviewed in (15)). In line with this, a study showed that termination commonly occurred at the midpoint between two origins if they had fired at similar times (18). Moreover, recent studies demonstrated that ubiquitylation of Mcm7 at the time of termination promotes replisome disassembly (19, 20), which indicated that replication termination could be as coordinated as replication initiation.

1.2 MITOSIS

The mitotic process can be categorized into four sub-phases, which are then followed by the division of the cytoplasm, called cytokinesis, of a single cell into two daughter cells. These four phases are called; prophase, metaphase, anaphase and telophase. During prophase the genetic material is condensed and reorganized into rod-like structures. The condensed DNA can be visualized in the light microscope as individual units known as chromosomes. Moreover, the nuclear envelope surrounding the DNA is broken down at the end of prophase. In metaphase, chromosomes align at the metaphase plate and microtubules of the spindle apparatus attach to the centromers of each chromosome. During anaphase, the sister chromatids separate and the spindles facilitate their migration to the opposite poles of the cell (Fig. 2). In telophase, the spindles are detached and degraded. The chromosomes de-condense and the nuclear envelop reforms, whereby the cell then undergoes cytokinesis. At the end of mitosis, one single cell has divided to generate two genetically identical cells. In contrast to meiosis, a special type of cell division described in the next section, generates genetically distinct cells through a process called recombination.

1.3 MEIOSIS

Meiosis is performed in diploid cells and specifically in germ cells of higher eukaryotes. In contrast to the two identical cells generated in mitosis, meiosis involves a pre-meiotic S-phase followed by two cell divisions. The two cell divisions are called meiosis I and II, whereby four genetically distinct haploid cells are generated. Thus meiosis is a specialized cell division process that reduces the number of chromosomes by half. An overview of meiosis and mitosis alongside each other is provided in figure 2.

Before meiosis begins the DNA of each chromosome is duplicated during pre-meiotic S-phase. Like S-phase in mitotic cells, the DNA of each parental chromosome is replicated

generating two identical sister chromatids that are held together through sister chromatid cohesion during pre-meiotic S-phase. During meiotic prophase homologous chromosomes begin to pair in a process called synapsis. This is accompanied by programmed DNA double-strand break (DSB) formation. The initiated DSBs are repaired preferentially by interaction with non-sister chromatids resulting in formation of crossovers, which can become physical chromosomal links called chiasmata. A tripartite structure called the synaptonemal complex (SC) forms between homologous chromosomes and mediates pairing of chromosomes. By the end of meiosis I, genetic recombination has taken place between the non-sister chromatids at the corresponding sites of crossovers and the homologs have been segregated into two separate cells. These cells then undergo meiosis II, which segregates the two sister chromatids and distributes them into a total of four haploid cells. The haploid cells generated during meiosis can in higher eukaryotes differentiate into gametes such as sperm and egg.

Prophase I takes the longest to complete and contain many of the events that are unique for meiosis. In paper I, we reveal and discuss new insights about Smc5/6 function during Prophase I of *Drosophila* female meiosis and thus further details are presented here. During early meiotic prophase Spo11 (**spo**rulation 11) generates transient DSBs from which crossovers can be formed. Around the same time synaptonomal complexes (SC) are assembled between homologous chromosomes (21-23). The tripartite SC structure consists of axial elements (AE) (also known as the lateral element), central elements (CE), and transverse filaments (TF) (24, 25). The stepwise assembly of the SC during prophase I is divided into four sub-stages known as: leptotene, zygotene, pachytene and diplotene. At the leptotene stage the AE are assembled on each chromosome in a homolog and serve as attachment points for TFs, which are assembled in-between the AEs during zygotene as homologs begin to pair. During this process the CE is assembled in-between the AEs and on the TFs. Crossover structures can then form between the non-sister chromatids and reside within the SC. Notably, there are differences between organisms in the timing of SC assembly in relation to DSB-induction and crossover-formation. Regardless of whether the SC is assembled before or after these events, the function of the SCs has been proposed to help stabilize sister chromatid linkages and possibly convert crossovers to functional chiasmata (26). During pachytene the SC is completely assembled and will disassemble at the end of pachytene or beginning of diplotene, whereby the chiasmata between non-sister chromatids remain. A subset of the crossovers will also result in a reciprocal exchange of similar DNA sequences between non-sister chromatids.

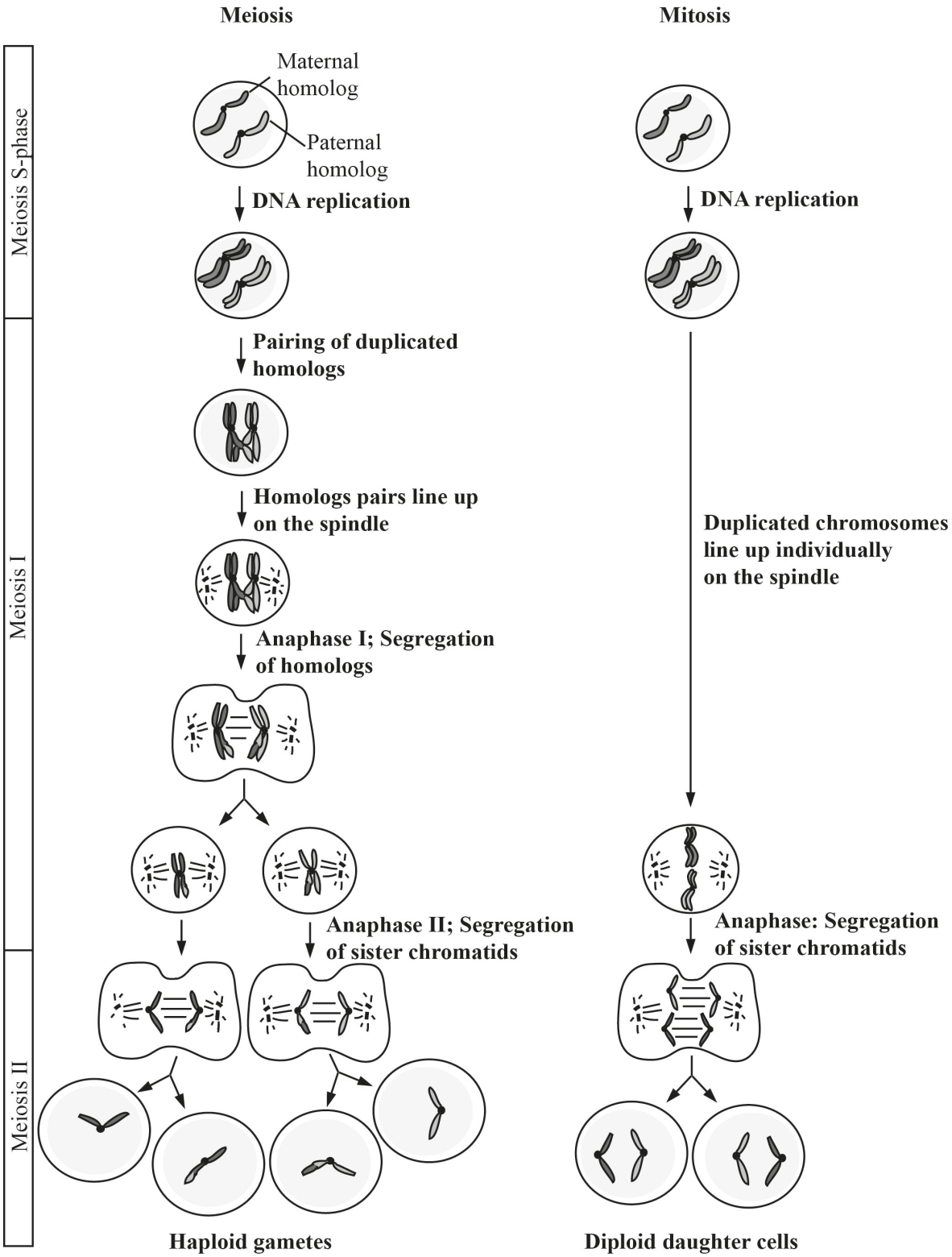

Figure 2. A comparative overview of meiosis and mitosis. Both meiosis and mitosis begin with DNA replication. For simplicity only one copy of paternal and maternal chromosomes are shown. Subsequent to S-phase, one cell division occurs in mitosis, whereas in meiosis the cell undergoes two divisions referred to as meiosis I and meiosis II. Moreover, in meiosis I chromosomes align together with their homolog via crossovers that form through recombination between non-sister chromatids, which in addition to allow proper segregation, leads to genetic diversity. At the end of meiosis, four genetically distinct haploids have been generated, whereas the end of mitosis generates two identical diploid cells.

With specific focus on *Drosophila* female meiosis and oocyte development (see also section 3.1.1), the assembly of the SC takes place during early pachytene before DSBs have been initiated by mei-W68 (a Spo11 ortholog) (22, 27). Repair of the DSBs take place mid pachytene, whereby the SCs gradually start to disassemble at late pachytene and onwards (28). The past two decades of research on *Drosophila* meiosis have revealed the identity and function of several proteins that form part of the SC (28-32). An overview of the currently identified proteins is provided in figure 3. Further insight into SC proteins has been contributed by the development of antibodies (27, 29, 30, 33). The AE in *Drosophila* oocytes is made up of ORD (**or**ientation disruptor) and C(2)M (**c**rossover suppressor on **2** of **m**anheim) , which assemble on homologous chromosomes (Fig. 3). Additionally, the AEs are also thought to be composed of proteins important for chromosome structure such as sister chromatid cohesion proteins (34). TFs then hold the AEs together and in *Drosophila* the TFs are made up of C(3)G (**c**rossover suppressor on **3** of **g**owen) dimers. Cona and Corolla localize to the CE and are important for stabilizing the c(3)G filaments. An antibody against Corolla was used in paper I to evaluate the number of cells with SC formation and progression in oocyte development. Notably, defects in any of the identified SC proteins are accompanied with reduced recombination due to diminished crossover formation (28-32), and defects in ORD and Corolla have been reported to lead to chromosome missegregation.

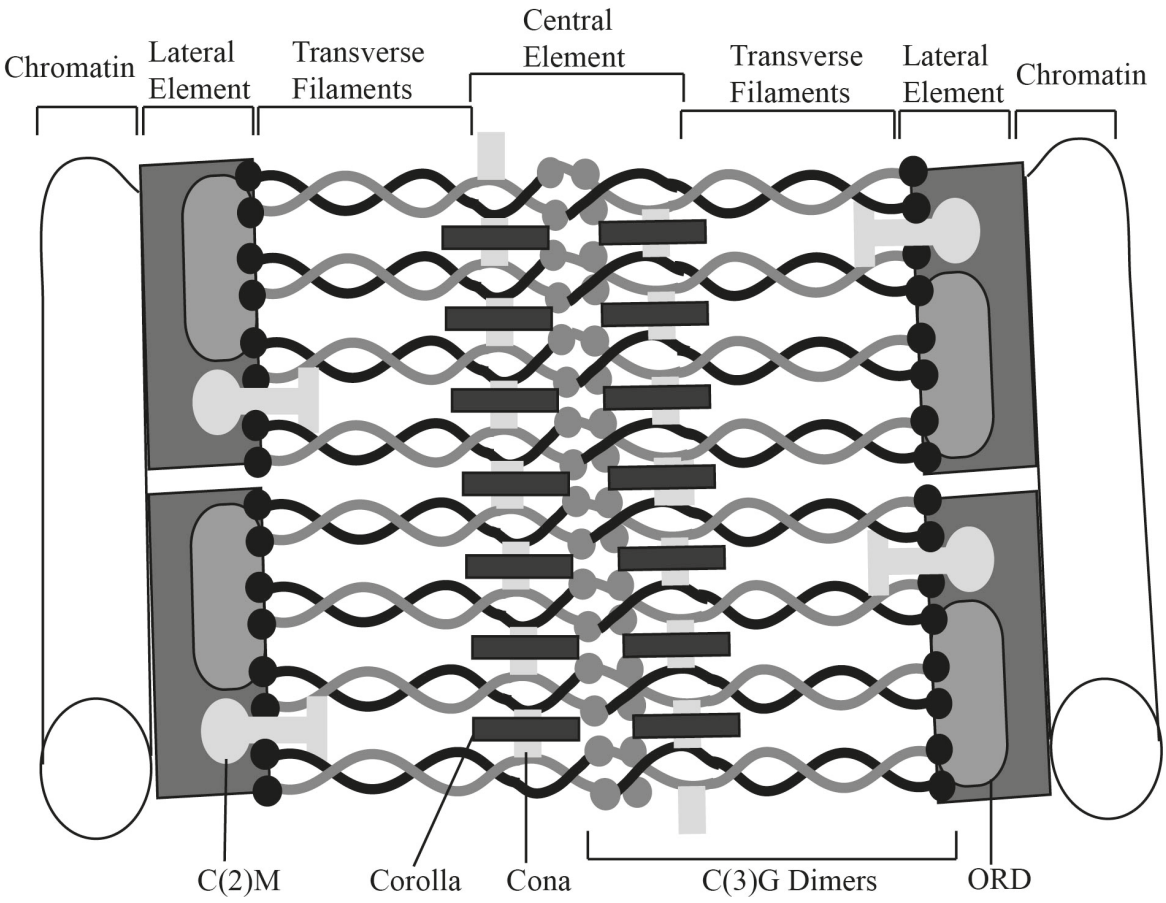

Figure 3. An overview of components in the synaptonemal complex (SC) of *Drosophila* oocytes. The figure has been adapted from Hemmer and Blumenstiel (2016). Axial (also called lateral) elements (AE) are established along the length of chromosomes. ORD and C(2)M have been identified as two of the AEs. The transverse filaments (TFs) function to connect the AEs of opposite sides and also serve as a scaffold, where the central element (CE) can assemble. C(3)G dimers form the TFs while Corolla and Cona (also known as Corona) localize to the central element to stabilize the TFs.

1.4 CHECKPOINTS AND DSB REPAIR VIA HOMOLOGOUS RECOMBINATION

Upon cell division eukaryotic cells respond to DNA damage by delaying cell cycle progression. This control mechanism, known as the cell cycle checkpoint, ensures proper division of the cell by giving the cells more time to process the irregularities. The following section has limited its description to processes and checkpoints that are relevant for paper I and II.

1.4.1 DNA DSB- and replication fork -repair via homologous recombination

DNA DSBs can be generated by exogenous (e.g. ionizing radiation) or endogenous (e.g. reactive oxygen species) agents. The response to DSBs prior to repair can be categorized into three events: sensing of the DSB, checkpoint activation, and modifying the break site. The latter includes processing of DSB ends and post-translationally modification of nucleosomes within the break region in order to facilitate repair.

In budding yeast, Mre11 (**m**eiotic **re**combination 11), Rad50 (**rad**iation sensitive 50), and Xrs2 (**X**-**r**ay **s**ensitive 2) form the MRX complex. MRX senses the DSB by binding to the broken ends (Fig. 5), whereby an ATM (**a**taxia-**t**elangiectasia **m**utated) ortholog called Tel1 is recruited in an MRX-dependent manner (35, 36). This is important for the DSB repair response, which among other things include an arrest in cell cycle progression in order to give the cell enough time to repair the DSB(s). Such an arrest can be mediated by a signaling cascade by Tel1 and Mec1 in which Rad53 is activated (37, 38). Moreover, Tel1 phosphorylates Rad9 and Chk1, which are part of this cascade and necessary for a Tel1 mediated cell cycle arrest (37).

Tel1 also phosphorylates histone H2A at the DSB site, which promotes the recruitment of additional repair factors (39). Phosphorylation of H2A at DSBs is an evolutionarily conserved response and can be used as a marker to assay for presence of DSBs (33, 40-43). Related to this, in paper I, a phosphorylated *Drosophila* histone H2A variant called γ-H2AV was used to quantify the presence of DSBs in the oocyte nucleus during pachytene.

DSBs can be repaired via non-homologous end joining (NHEJ) or homologous recombination (HR). The choice of DSB repair pathway is mainly controlled by the cell cycle stage at which the DSB takes place (reviewed in (44)). DSB repair via NHEJ involves the ligation of two broken ends, whereas DSB repair via HR involves template-dependent exchange of DNA sequences. DSB repair via HR can be observed during various processes within the cell such as; S-phase replication and fork progression, sister chromatid repair during G2, in budding yeast mating-type switch in budding yeast, and pairing of homologous chromosomes during meiosis I. An overview of the HR process in budding yeast following a DSB has been provided in figure 4.

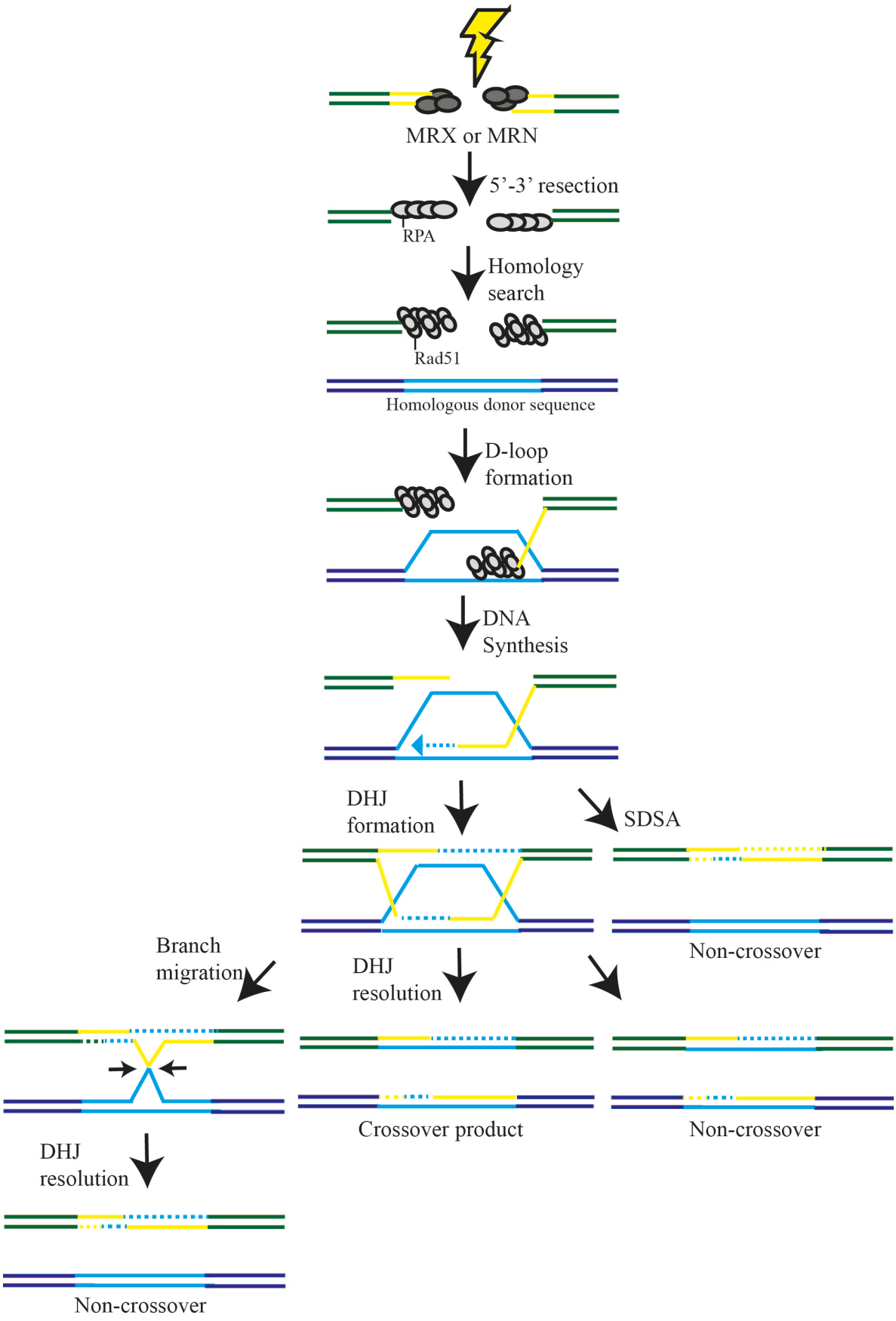

Figure 4. An overview of the HR process and products. The process is described in the text.

After a DSB has taken place, HR initiates with the nuclease activity of Mre11, which resects the 5'-3' end of DNA at the break site together with Sae2 (**s**porulation in the **a**bsence of spo **e**leven 2) forming a single-stranded overhang (35). The resection is continued together with the activity of Exonuclease 1 and a helicase-endonuclease activity of the STR-Dna2 complex ((Sgs1 (**s**low **g**rowth **s**uppressor 1)-Top3 (**top**oisomerase 3)-Rmi1 (**R**ecQ **m**ediated genome **i**nstability 1) – **DNA** synthesis defective 2)) (45). A ssDNA protein called replication protein A (RPA) then coats the processed DNA and prevents the ssDNA from being degraded or from forming secondary structures. RPA needs to be replaced as it otherwise inhibits strand exchange, which is a process downstream. RPA is substituted by a recombinase called Rad51, whereby a heterodimer of Rad55 and Rad57 helps Rad51 to compete with RPA for ssDNA binding (46). In budding yeast RPA substitution with Rad51 is further mediated by Rad52 activity (47), which is augmented by Rad59 (48). Notably, *Drosophila* lacks a Rad52 homolog and human cells rely on BRCA2 (**br**east **ca**ncer 2) while also having a Rad52 homolog (49). Differences in HR proteins such as this also exist at later steps of the HR process described below. However, the overall progression of the HR process is thought to be evolutionarily conserved.

As Rad51 is assembled in the place of RPA it forms a nucleoprotein filament complex with the DNA (50), which can pair with a homologous sequence once it is found via a process called homology search (Fig. 4) (51). The nucleoprotein filament can then initiate a strand exchange with the donor DNA. This causes a displacement of the donor DNA's complementary strand and a displacement loop (D-loop) is formed. The broken 3' end is then primed for DNA synthesis, which causes the D-loop to extend. Different outcomes resulting in either crossover or non-crossover products can then be generated (Fig. 4) (reviewed in (52) and (53)). One outcome could be that the second DSB end aligns with the extended D-loop to form double holliday junctions (dHJ). These can be resolved by the resolvases such as Mus81-Mms4 (**m**ethyl methane sulfonate and **u**ltraviolet **s**ensitive - **m**ethyl **m**ethanesulfonate **s**ensitivity) complex, which either produces a crossover or non-crossover product from the dHJs. This repair type, even though crossover products only form a portion of the time, is important during meiosis in order to produce genetic variation through exchange of genetic sequences between homologs. Conversely, a non-crossover product is preferred for repair of mitotic DSBs in order to maintain identical genetic material. A non-crossover product can be formed by the migration of the two branches of the dHJ toward each other, whereby their dissolution upon convergence can be promoted by the action of the STR complex. A non-crossover product can also be generated through the suppression of dHJ formation, which is the case for the synthesis-dependent strand-annealing (SDSA) pathway. In SDSA the invading strand is displaced after DNA synthesis (Fig. 4) and anneals with the second DSB end.

Restarting stalled replication forks during S-phase requires HR (54, 55). Stalling occurs when the fork pauses at an unintended site. Such fork pausing can occur due to DNA lesions on or in the template (e.g. thymidine dimers or DNA alkylation), whereby fork restart is important for replication completion in a timely manner before cell division. The molecular process of

fork restart is still not fully elucidated. Many models advocate unwinding of a stalled fork to create a four-stranded DNA structure reminiscent of a HJ, which are thought to be created through a process called replication fork regression (56, 57). A possible way that regression could restore fork progression when the leading strand replication encounters a lesion is depicted in figure 5. In the depicted scenario the lagging strand has progressed further in replication than the leading strand, whereby fork regression can facilitate strand-switch synthesis. This enables the leading strand to use the lagging strand as a template for the region, which is blocked by the lesion. Upon reversal of the fork regression replication can restart on the leading strand beyond the lesion without leaving a gap on the leading strand opposite to the lesion.

Failure to restart stalled forks can result in fork collapse. Ideally, this would be repaired by a sister chromatid, but this does not become available until replication has completed. A collapsed fork is thought to result in a DSB with a single end (58), which can be visualized in figure 4 by imagining the absence of a second DSB end at the DNA synthesis step. As the second DSB end is unavailable, there is a need to search for another (less) homologous duplex sequence. Upon strand invasion a processive replication fork is established such that DNA synthesis proceeds until the chromosome end (59). This process is called break-induced replication (BIR). In contrast to the HR paths depicted in figure 4, which only repairs or replaces sequences of a small section near the DSB site, BIR changes the sequence from the DSB site until the chromosome end.

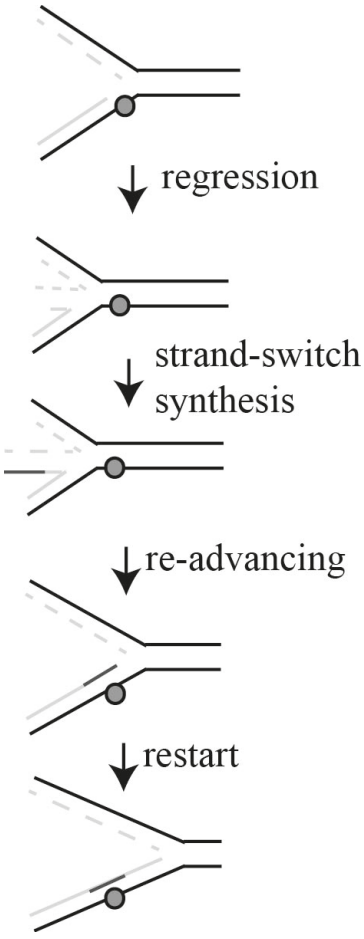

Figure 5. Model of how fork regression facilitates fork restart when the leading strand encounters a lesion. Here replication of the leading-strand encounters a lesion, which stalls the fork. The lagging strand has proceeded beyond the leading strand and can subsequent to fork regression be used as a template in strand-switch synthesis for subsequent fork progression. The fork then reverses the regression in a re-advancing step, whereby a newly synthesized fragment opposite to the lesion has now been generated. Replication of the leading strand can now restart and progress.

1.4.2 Pachytene delay

Pachytene delay, also called the pachytene checkpoint arrest, takes place in meiosis. Cells with defects in meiotic recombination, delay in pachytene of prophase I. This is visualized by either the assembly state of SC (section 1.3), or in *Drosophila* it can also be indicated by the delay in oocyte selection (60, 61). DSB –dependent and -independent pathways have been identified, which can delay cell cycle progression during pachytene. This delay is thought to provide cells with more time to respond and resolve irregularities in the meiotic recombination process, which is required both to facilitate genetic variation and hold the homologs together for proper chromosome segregation at anaphase I. As stated, meiotic recombination consists of several steps such as synapsis, SC assembly, crossover formation, SC disassembly, and crossover resolution. As these steps are tightly interlinked, it has been difficult to discern which defect(s), belonging to one or several of these steps, trigger pachytene delay.

The DSB-dependent pachytene delay is dependent on mei-41 (**mei**otic 41), an ATR homolog in *Drosophila*, and has been observed in DNA repair mutants (e.g. *rad51*) (62). The DSB-dependent pathway responds to the accumulation of unrepaired DSB by arresting cell cycle progression, but may also be directly involved in DSB repair. In line with this, *mei-41* mutants exhibit unrepaired DSBs and a reduction in crossovers (63).

A DSB- and mei-41 –independent pathway for pachytene delay has also been identified. This pathway is mediated by Pch2 (**p**achytene **ch**eckpoint 2), whereby it has been unclear what defect(s) in meiotic recombination activates this pathway. Studies in budding yeast and in *Caenorhabditis elegans* indicated that irregularities in synapsis caused a Pch2 mediated pachytene delay (64, 65). However, in *Drosophila* Pch2 dependent pachytene arrest is believed to provide the meiotic cells with more time to allow the formation of crossovers (60, 66). In line with this, a c(3)g mutant, which had defective synapsis did not induce pachytene arrest (60). Moreover, mutants with already reduced crossover formation achieve even fewer crossovers when Pch2 is mutated (60, 66).

2 THE SMC5/6 COMPLEX

The SMC family consists of the three complexes: Cohesin, Condensin, and Smc5/6. During cell division Cohesin and Condensin are known to hold together sister chromatids for correct segregation and enable chromosome condensation, respectively (67-69). The main function of Smc5/6 is still elusive and has not yet been given a name based on a specific primary function. Smc5/6 consists of Smc5 and Smc6 making up the backbone of the complex, and six associated non-Smc elements (Nse) (Fig.6). The components of the Smc5/6 complex are summarized in table 1 and from hereon the subunits will be referred to by their name in budding yeast. Lehmann and colleagues discovered the *SMC6* gene, at that time termed *RAD18*, through a screen for radiation sensitivity in fission yeast (70). Remainder components of the complex were identified via a chromatin immunoprecipitation experiments, which indicate that it forms an octameric complex (71-75). The complex has not

yet been crystallized, whereby the current predicted structure of Smc5/6 comes from domain predictions based on amino acid sequence and biochemical studies.

Saccharomyces cerevisiae	*Schizzosaccharmyces pombe*	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Xenopus laevis*	*Homo sapiens*
Smc5	Smc5/Spr18	SMC-5	SMC5	SMC5	SMC5
Smc6	Smc6/Rad18	-	SMC6/CG5524	SMC6	SMC6
Nse1	Nse1	-	CG11329	NSE1	NSE1
Mms21/Nse2	Nse2	-	CG13732 & CG15645	NSE2	NSE2
Nse3	Nse3	-	Mage	-	MAGE-G1
Nse4	Nse4/Rad62	-	CG13142	-	NSE4A & NSE4B/EID3
Nse5/YML023c	Nse5	-	-	-	-
Nse6/Kre29	Nse6	-	-	-	-

Table 1. Subunits of the Smc5/6 complex in different species. Smc (Structural maintenance of chromosomes). Nse (Non-smc element). Kre (Killer toxin resistant). Mage (Melanoma-associated antigen). Rad (Radiation sensitive). Mms (Methyl methane sulfonate sensitivity). CG (Computed Gene). EID (E1A-like Inhibitor of Differentiation).

The predicted domain structure of Smc5 and Smc6 appear very similar to each other. Each of them has a N- and C -terminal globular head. The globular heads interact to form an ATP binding site when the coiled-coil domains are folded via their hinge domain (Fig. 6). Smc5 and Smc6 form a sub-complex with Nse2, which is the only Nse protein that has a confirmed enzymatic activity. The SP-RING domain of Nse2 confers sumoylation activity, which is important for DNA repair efficiency (76, 77). Notably, it was shown that the sumoylation activity was dispensable as long as Smc5/6 could still form a complex with Nse2. This implied that Smc5/6 performs essential roles other than sumoylation (77). Nse1 contains a variant RING (**r**eally **i**nteresting **n**ew **g**ene) domain that is characteristic for ubiquityl-ligases, but has so far not been shown to have any enzymatic activity. Nse3 is a protein with homology to the Melanoma Antigen family, which are expressed and recurrent in tumours (78-80). Nse4 is a member of the Kleisin protein family and related to a transcription repressor called EID (**E**1A-like **i**nhibitor of **d**ifferentiation). Together Nse1, Nse3, and Nse4 form a heterotrimeric sub-complex (Fig. 5) (79, 81, 82).

Biochemical and genetic studies have shown that there are differences in the essentiality of the subunits and how the subunits of Smc5/6 arrange themselves in the complex. In fission

yeast (*Schizosaccharomyces pombe*) Nse5 and Nse6 are thought to bridge the globular heads of Smc5 and Smc6, whereas they are associated to the hinge-domain of Smc5 and Smc6 in budding yeast (81, 83). Furthermore, Nse5 and Nse6 are essential in budding yeast, but not in fission yeast. Notably, Nse5 and Nse6 still remain unidentified in many eukaryotes such as humans. The sequence of these proteins is not well conserved among organisms, which have made it difficult to identify homo- or ortho –logs. Recent findings in mice and plants, however, have implicated proteins that could be the equivalents of Nse5 and Nse6 (84, 85). This indicates that all Smc5/6 subunits might be conserved and function as an octamer.

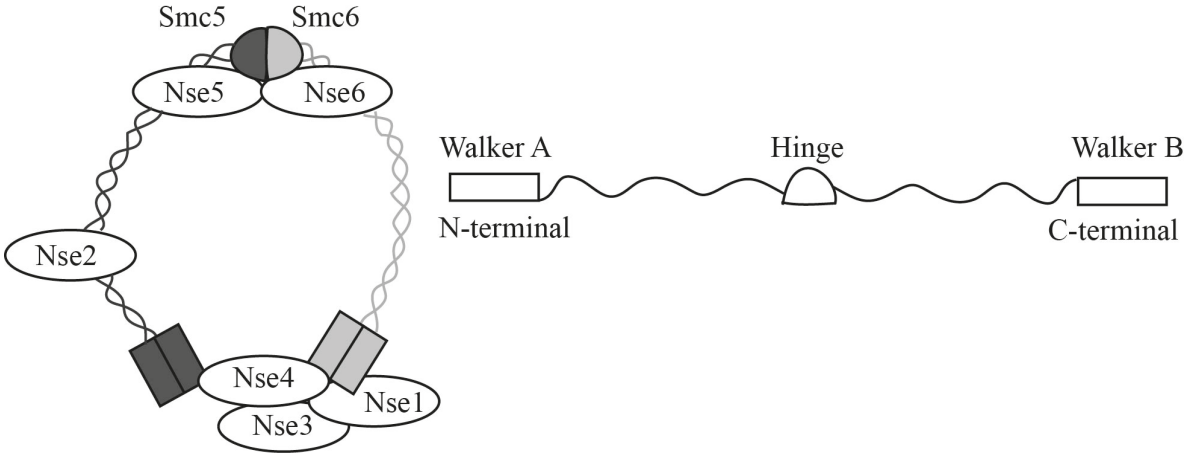

Figure 6. Overview of the Smc5/6 complex in budding yeast. Smc5 and Smc6 protein are very similar to each other. Their overall structure can be viewed on the right side. The N- and C- terminal part of Smc5 and Smc6 contain a Walker A and B domain, respectively. The N- and C-terminal end associate with each other to form a functional ATPase head, whereby the coil regions that connect to the midway hinge domain form a coiled-coil structure. Together Smc5 and Smc6 make up the backbone of the complex, to which six additional Non-Smc elements (Nse) can bind.

2.1 SMC5/6 IN DNA DOUBLE-STRAND BREAK REPAIR AND HOMOLOGOUS RECOMBINATION

Smc5/6 was originally identified as a necessary component needed to survive ionizing radiation. Radiation is an exogenous agent that causes DNA DSBs. The complex has been found to localize to DNA DSBs (86, 87), whereby impaired DNA repair and chromosomal fragmentation have been observed when components of Smc5/6 are mutated or absent (70, 77, 88-92). Initial genetic analysis placed Nse1, Smc6, and Nse2 in the pathway of HR (70, 71), whereby it is through HR that Smc5/6 is thought to mediate DSB repair. How Smc5/6 operate in the pathway of HR has not been fully elucidated and the precise role of Smc5/6 in this process is still uncertain.

It has been proposed that Smc5/6 recruits Cohesin to DSBs, and thereby hold the sister chromatids in close proximity to each other at the break site (93, 94). This would then mediate more efficient HR and DSB repair. However, the recruitment of Cohesin to DSBs by Smc5/6 is not preserved in budding yeast, although the complex is still required for efficient repair (77, 95).

In contrast to mediating HR, Smc5/6 has also been shown to have an anti-recombinogenic function (94, 96-98). Cells lacking Smc5/6 function exhibit hyper-recombination and gross chromosomal rearrangements, whereby abolishing recombination alleviated these phenotypes.

The hyper-recombination phenotype can be connected to a function of Smc5/6 resolving recombination intermediates. Such intermediates may appear at the telomeres, whereby the resolution of them is important for slowing telomere shortening and cell senescence after each round of replication (99, 100). Moreover, meiotic cells lacking functional Smc5/6 were shown to accumulate a type of recombination intermediates called joint molecules. Unresolved joint molecules were accompanied with chromosome segregation defects. Restoration of Smc5/6 function allowed these intermediates to be resolved and the chromosome segregation defect to be rescued (101-103). A possible way that Smc5/6 could mediate resolution of recombination intermediates is by interaction with the STR helicase-topoisomerase complex. Smc5/6 interaction with STR was recently shown to promote the sumoylation of STR members, which enhanced the ability of STR to assist in removal of recombination intermediates (104).

Altogether, Smc5/6 enables HR to be performed more efficiently at DSBs through the recruitment of Cohesin, prevent gross chromosomal rearrangement by suppressing aberrant recombination, and contribute to the resolution of recombination intermediates. In yeast, abolishing HR does not cause lethality, suggesting that the essential function of Smc5/6 is independent of its function in HR.

2.2 SMC5/6 IN REPLICATION FORK STABILITY AND PROGRESSION

In absence of Smc5/6, forks progression is slower and stalled replication forks are not restarted correctly, whereby under-replication is observed as well as formation of detrimental recombination structures (92, 105-107).

One way that Smc5/6 is thought to mediate replication fork progression is by recruiting or modulating proteins important for restarting stalled- and repairing collapsed -forks. Smc5/6 is expressed during S-phase and has been demonstrated to localize to stalled forks (55, 87, 106, 108-110). At stalled forks Smc5/6 has been shown to interact and modulate the activity of Mph1, which is a protein involved in the initial process of fork restart called replication fork regression (105, 111). In absence of Smc5/6, this process is unregulated and Mph1 is thought to generate detrimental recombination intermediates. Rad52 is another protein important for fork restart (112). It was shown to be recruited to stalled forks in a Smc5/6-dependent manner (55). Once Rad52 is recruited it is thought to facilitate nascent strand-exchange, which is required for fork restart. Rad52 is also recruited upon fork collapse, but the recruitment of Rad52 under this circumstance was shown to be Smc5/6-independent while Smc5/6 was still required for the repair of the resulting lesion (98).

Another way by which the Smc5/6 complex has been suggested to mediate replication fork progression is through promoting fork rotation (113). In this model, the Smc5/6 complex

works alongside enzymes called topoisomerases. These enzymes resolve topological entanglements such as DNA supercoils or sister chromatid intertwinings that are generated by progression of the replication fork and fork rotation, respectively. Topoisomerase 2 resolves sister chromatid intertwinings generated behind and in front of the replisome, whereas Topoisomerase 1 (Top1) resolves positive supercoils that are generated ahead of the replisome. In absence of Top1 superhelical tension accumulates and the replisome is hindered resulting in a replication delay, which is visible for long chromosomes. Intriguingly, absence of Smc5/6 caused a similar replication delay of long chromosomes as seen when Top1 is absent (113). This indicated that resolution of replication-induced superhelical stress is important for replication fork progression and that Smc5/6 could be involved in this process.

2.3 SMC5/6 IN CHROMOSOME SEGREGATION

During mitosis the chromosomes align and microtubules are attached to them, which facilitate their segregation and distribution into the daughter cells. If this process is impaired cells can acquire chromosomal abnormalities such as; irregular number of chromosomes and/or chromosome fragmentation followed by micronuclei formation. These abnormalities are often accompanied by absence of functional Smc5/6 (1, 2, 92, 114). Gallego-paez *et al.* (2014) showed that mitotic chromosomes in human cells exhibited lagging and anaphase-bridges, whereby they noted that the latter likely generated breakages and chromosome fragments that could form micronuclei (92). Similar formation of micronuclei or anaphase-bridges in absence of functional Smc5/6 has also been reported in budding yeast and mouse embryonic stem cells (115, 116).

One way in which Smc5/6 might influence chromosome segregation is by proper activation of the spindle assembly checkpoint (SAC), which function to prevent chromosomes from segregating before microtubules have been properly attached to them. In line with this, Pryzhkova and Jordan (2016) showed that Smc5/6 localized to spindle poles in mouse embryonic stem cells during mitosis and that Smc5/6 co-localized with a SAC protein called Mad2 (**m**itotic **a**rrest **d**eficient) (116). Mad2 levels decreased upon Smc5 depletion, whereby the authors suggested that SAC function had been disrupted or prematurely satisfied prior to chromosome segregation.

Another way by which Smc5/6 could influence chromosome segregation is through direct interaction with microtubules, which was demonstrated by Laflamme *et al.* (2014) (117). In this study Smc5 was shown to facilitate more efficient bundling of tubulin and that Smc5 could bind both tubulin and DNA simultaneously *in vitro*. Moreover, Smc5 binding with tubulin was visualized *in vivo* in dividing yeast cells.

Smc5/6 has also been shown to aid chromosome segregation by indirect means, which is demonstrated by its role in resolution of recombination intermediates or other entities that may impede chromosome segregation if left unregulated and unresolved (101, 102, 104, 114, 118, 119).

2.4 SMC5/6 AT HETEROCHROMATIN

DNA is wrapped around nucleosomes made of histone octamers. This wrapping can be adjusted such that some regions are more open and accessible for DNA processes such as replication and transcription. These regions are called euchromatin. Conversely, some regions are more closed and these are called heterochromatin. The inaccessible content in heterochromatin can be referred to as silenced or transcriptionally repressed. Such regions are also often accompanied with certain post-translational modifications of histones such as methylated lysine 9 of histone H3 (H3K9me). Notably, budding yeast lack H3K9me and its transcriptionally repressed regions are therefore referred to as heterochromatin-like, but for simplicity it will be referred to as heterochromatin hereafter. Common for yeast and many other organisms is that heterochromatin is typically found at centromeres, ribosomal DNA (rDNA), and telomeres.

Smc5/6 has been shown to localize to heterochromatic regions and the heterochromatin marker H3K9me (87, 92, 98, 119-123). Additionally, the complex has been proposed to perform a function in transcriptional repression important for the formation of the XY body (121). In line with this, Smc5/6 was demonstrated to mediate telomeric silencing in budding yeast (124, 125) and repress Hepatitis B virus gene expression (126). These observations suggest that Smc5/6 might contribute to heterochromatin establishment and transcriptional gene regulation.

Apart from transcriptional repression, Smc5/6 has also been shown to localize to heterochromatin to suppress recombination such that DNA repair via HR can take place outside of heterochromatin (89, 127). As Smc5/6 localizes to various heterochromatic regions, it would suggest that Smc5/6 also has a general role in heterochromatin maintenance by protecting these regions from aberrant recombination.

2.5 SMC5/6 IN DISEASE AND DEVELOPMENT

Two clinical case studies have been reported of patients with mutations in Nse2 or Nse3, respectively (1, 2). Patients with compound heterozygous mutations in Nse2 suffered from primordial dwarfism, primary ovarian failure, and extreme insulin resistance. Primary cells from these patients exhibited increased micronuclei formation. This phenotype could only be rescued by expression of Nse2 with sumoylation activity. In comparison, patients with Nse3 missense mutations suffered from acute respiratory distress syndrome in early childhood followed by death during infancy. This was attributable to combined T and B cell immunodeficiency, which led to increased susceptibility to viral infections in the lungs. Cells from these patients also exhibited reduced stability of Smc5/6, which were accompanied with chromosome rearrangements, micronuclei, and defective homologous recombination. These defects have been previously described to cause cell death in other organisms and cell types, which lacked functional Smc5/6.

Intriguingly, the Hepatitis B virus, which infects liver cells, was shown to target Smc5/6 for degradation in order to enhance its ability to propagate and sustain infection (128).

Inactivation of the viral gene product responsible for targeting Smc5/6 for degradation led to transcriptional repression of the episomal genes from the Hepatitis B virus, which impaired its propagation (126, 128). This implicated Smc5/6 in transcriptional repression and also suggested that Smc5/6 acted as a host anti-viral factor. Prolonged infection with Hepatitis B has been associated with an increased risk of developing cirrhosis and hepatocellular carcinoma (129, 130), whereby the liver acquires too much scar tissue to function normally and cancer, respectively. Thus, absence of Smc5/6 may give rise to cirrhosis and hepatocellular carcinoma.

Smc5 mutations have also been implicated in another form of cancer, where it was associated with brain metastases (3). However, not all cancer cells benefit from mutated or absent Smc5/6. It has previously been shown in a human cancer cell line with inactive telomerase that Smc5/6 mediates alternative lengthening of telomeres (ALT) (131). This process involves HR at the telomeres in order to lengthen them, which slows down telomere shortening and cell senescence after each round of replication. Depletion of Smc5/6 inhibited telomere HR and abolishing the SUMO ligase activity resulted in telomeres not localizing to sites called promyelocytic leukemia bodies, which is where ALT takes place.

3 ASPECTS OF THE MODEL ORGANISMS

3.1 FRUIT FLIES

The fruit fly, *Drosophila melanogaster*, has been used in research for over a century to study various biological phenomena. As a model organism *Drosophila* is easy to handle, inexpensive to maintain, and has a short generation time. Moreover, the *Drosophila* genome is distributed across four chromosomes, which has been sequenced and annotated. This makes it more straightforward to compare and correlate findings that may be present in other animals and organisms. In line with that, a study found a significant amount of matching human disease genes to be present in the *Drosophila* genome (132).

The ability to quickly generate stocks that combine several traits of interest is an attractive feature of *Drosophila*. This is achieved by crosses, which have been facilitated by the use of markers in combination with one or several genes of interest. These markers are visible on the fly (e.g. wings, eyes, or limbs), whereby genes whether natural or ectopically introduced can be tracked. Additionally, *Drosophila* males have a negligible recombination rate among their sperm, whereby recombination occurs principally in the egg of the females. This aspect further enables consideration on how to set up crosses and anticipate their outcome.

An alternative way of maintaining genes of interest is through the use of balancer chromosomes, which can be stably maintained throughout generations. The balancer chromosome usually replaces one of the two homologous chromosomes and presents a powerful tool in fly genetics. Most balancer chromosomes contain recessive lethal or sterile mutations, which renders them homozygous lethal i.e. flies that carry two copies of the

balancer chromosome are unviable (133). Moreover, the balancer chromosomes contain inversion breakpoints preventing recombination and dominant or recessive markers. Thus a fly stock with a balancer chromosome and a corresponding regular chromosome with the gene(s) of interest can be maintained and mate amongst themselves without the gene(s) of interest being lost or altered through recombination.

3.1.1 Oocyte development in *Drosophila*

Egg cell development, also known as oogenesis, has been favorable to study in *Drosophila*, as they are readily visible under the light microscope. Moreover, the ovaries contain oocytes (egg cells) arranged according to increasing age and maturity in an array called the ovariole (Fig. 7). Each ovary contains about 15-20 ovarioles, which have oocytes from prophase I to metaphase I. Remainder stages are completed after the egg enters the oviduct.

Development begins at the anterior compartment called the germarium, which is furthest away from the oviduct. The germarium can be further subdivided into three regions (Fig. 7). In region 1, also called the pre-meiotic region, a germ line stem cell divides asymmetrically to generate a new stem cell and a cystoblast (134). The cystoblast then undergoes four divisions without complete cytokinesis, which by region 2a forms a 16-cell cyst that are interconnected through so-called ring canals. In region 2a the 16-cell cyst enters early pachytene, which in *Drosophila* is accompanied with DSB initiation by mei-W68 and SCs assembly in up to four cyst cells out of the 16-cell cyst (22, 27, 28, 31). The two cyst cells with synaptonemal complexes are denoted as pro-oocytes, whereby one will be selected to become the oocyte by region 3 (60). The 16-cell cyst migrates to the posterior of the germarium, whereby repair of the DSBs begin in region 2b and completes in region 3 (62). The latter is also called stage 1 and in this stage the oocyte should have localized to the posterior. The other pro-oocyte alongside the remaining 14 cyst cells will begin to differentiate into nurse cells. Moreover, follicle cells now enclose the nurse cells and the oocyte in a follicle, which will bud off to form stage 2 in oogenesis.

During development the nurse cells will undergo endo-duplication and acquire a polytene nucleus. The role of the nurse cells in egg development is to metabolically support the oocyte with mRNA and proteins. This is possible through the ring canals, where the nurse cells can expel their cytoplasm into the oocyte in a process called dumping (135). The follicle cells will also undergo endo-duplication as the oocyte matures, whereby specific sections of the DNA corresponding to the chorion genes can be re-duplicated up to 80-fold relative to genomic DNA (136). The chorion genes encode eggshell proteins, whereby defects in these genes or in the endo-duplication of follicle cells cause a thinning of the eggshell and female sterility.

In stage 3 the oocyte progresses to late pachytene, whereby oocyte nuclei will begin to condense into a compact structure called the karyosome. (137). Shortly thereafter SC disassembly initiates, but will not complete until around stage 6 (28, 138). As development continues the compartment of the oocyte nucleus will become larger, whereby it will take up

approximately 50% of the egg size by stage 10. Furthermore, by stage 10 the karyosome will have moved into a dorsal position along with a vesicle surrounding it called the germinal vesicle, which contain proteins and mRNA important for karyosome maintenance (Fig. 7).

A protein named Gurken is important for the coordination between the oocyte and the follicle cells in establishing a dorsal-ventral axis. Notably, embryos inherit the established dorsal-ventral axis, whereby a dorsal-ventral polarity defect can manifest in embryogenesis if the axis is improperly established during oogenesis. Such a situation can arise in the presence of unrepaired DSBs in the oocyte nucleus followed by activation of the mei-41 checkpoint (section 1.4.2), which disrupts Gurken expression (139, 140).

During stage 10 the karyosome will de-condense in order to facilitate transcription (137, 141), but will re-condense by stage 12-13 before the nuclear envelope breaks down (137). At this stage follicular and nurse cells undergo developmentally regulated cell death. In nurse cells this is preceded by cell shrinkage and rapidly dumping its cytoplasm into the oocyte compartment via the ring canals.

At stage 13, the oocyte nucleus transitions from prophase I to metaphase I. This transition occurs around the same time that nuclear envelope breakdown takes place and is completed by stage 14, which is when the oocyte arrests in metaphase I. Remainder stages of meiosis I and II are completed upon rehydration and mechanical stress that are exerted as the egg enters the oviduct.

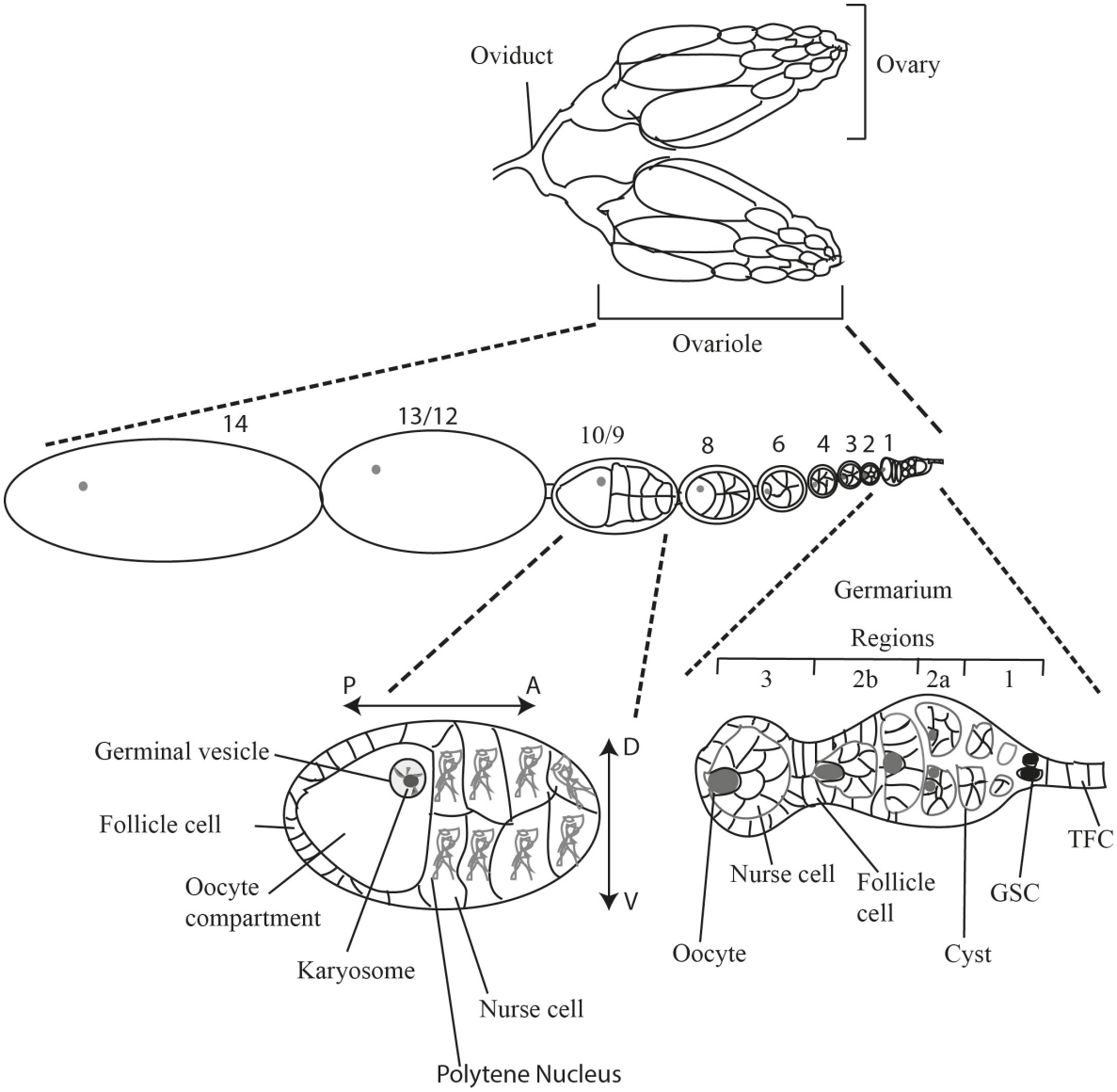

Figure 7. An overview of oogenesis in *Drosophila*. Ovaries contain about 15-20 ovarioles, which have oocytes of increasing maturity and meiotic progression starting from the germarium until stage 14. For simplicity some stages of oogenesis have been skipped and the nuclei of the follicle cells are not drawn. The germarium has an anterior tip consisting of terminal filament cells (TFC). Posterior to the TFCs the germarium can be divided into 3 regions. In these regions meiosis initiates and a more thorough description of events involved in this process is described in the main text. SCs assemble in up to four pro-oocytes in region 2a and programmed DSBs are induced by mei-W68 shortly thereafter. By region 3 DSBs in the oocyte should have been repaired and one of the pro-oocytes will become the oocyte while the others pro-oocytes and cysts become nurse cells. By stage 3 the oocyte nucleus condenses into a compact structure called the karyosome, which by stage 10 occupies a dorsal (D) position and de-condense briefly for transcription. A germinal vesicle containing proteins as well as mRNA important for karyosome maintenance surrounds the oocyte nucleus. An axis for anterior (a) and posterior (p) is shown along with an axis for dorsal (D) and ventral (V). By stage 12 and onwards both follicle- and nurse cells undergo developmentally regulated cell death. Prophase I takes place between region 2a in the germarium until stage 13. By stage 13 the oocyte begin transition into metaphase I. By stage 14 the transition is complete and the oocyte arrests until it is rehydrated and exposed to mechanical stress by the oviduct, which initiates completion of the remainder stages of meiosis I followed by meiosis II.

3.1.2 Early embryonic development in *Drosophila*

A female can lay >75 eggs per day and up to 2000 eggs in their lifetime (142), whereby development of the embryo takes place outside of the mother. Embryonic development, also called embryogenesis, can in simplified terms be divided into three subsections: rapid nuclear divisions (also known as cleavage cycles), syncytial blastoderm, and gastrulation. Here early embryonic development, also sometimes referred to as pre-gastrulation, is defined as the first two mentioned and is the focus of this section. Gastrulation and onwards are considered to be later parts of embryonic development, which include organ formation among other processes.

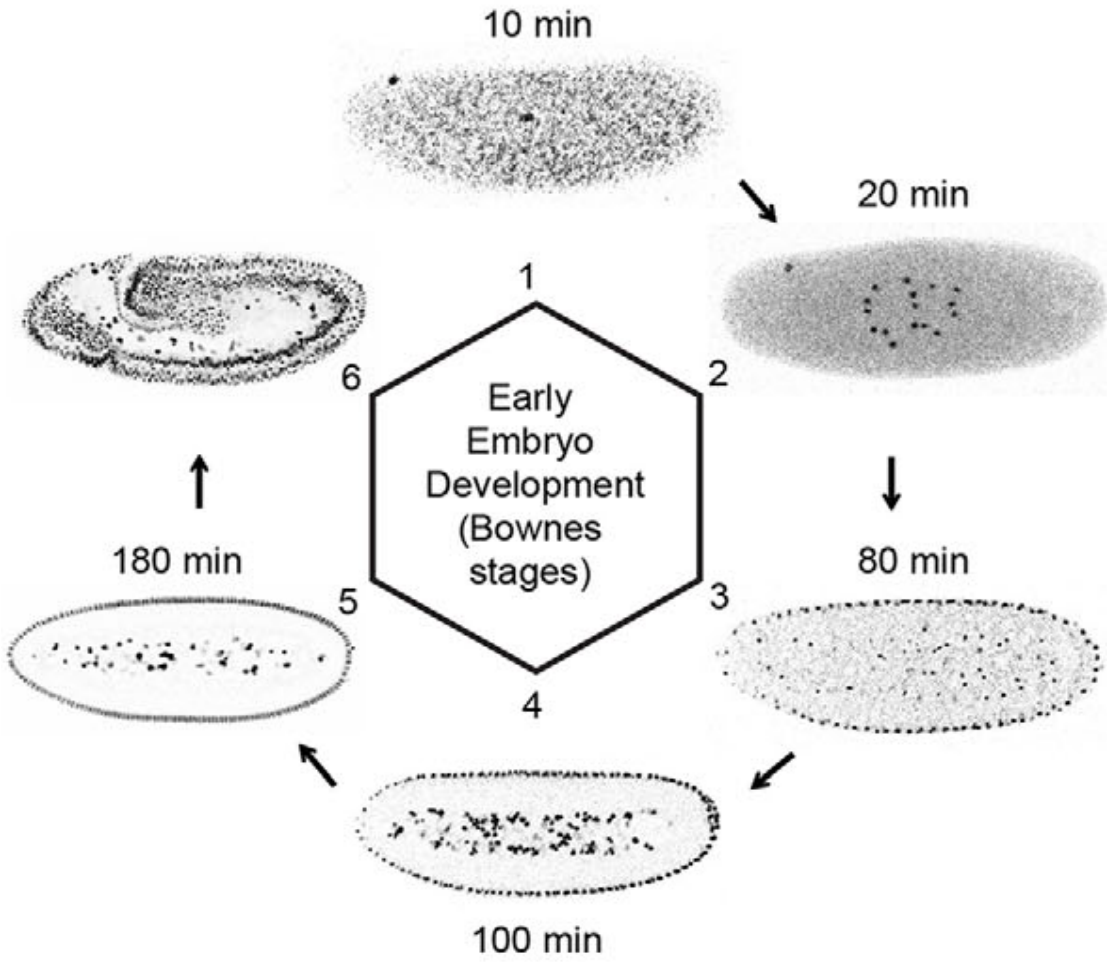

Figure 8. Overview of early embryonic development in *Drosophila* as defined by Bownes stages 1-6. Picture taken from *Tran et al.* (2016). The picture was generated from imaging data of embryos fixed and DNA-stained with propidium iodide at different time points, which are depicted alongside the annotated Bownes stages. The DNA can be seen as black dots. Starting from the zygotic nucleus the embryo undergoes rapid nuclear divisions without intervening gap phases, whereby they cycle between M- and S-phase. After Bownes stage 3 and after the 8th nuclear division cycle, majority of nuclei move to the periphery or surface, whereas some nuclei stay behind in the center. The nuclei, which remain at the center, are called yolk nuclei. As seen in Bownes stage 4 and 5, the yolk nuclei form a defined cluster in the center leaving some space between themselves and the surface nuclei. Towards the end of Bownes stage 4, damaged nuclei sink down from the surface and join the mass of yolk nuclei in a Chk2-dependent process called nuclear fallout. The removed nuclei are replaced by another round of replication by their neighboring nuclei. A membrane begins to form around the surface nuclei, whereby cellularization of the nuclei is initiated and acquire a more elongated than spherical form. Cellularization does not complete until Bownes stage 6, which is when the surface nuclei invaginate (fold inwards) and gastrulation initiates.

Early embryogenesis begins with the zygotic nucleus present in the egg. A series of rapid division cycles also known as cleavage cycles take place, whereby the nuclei alternate between S- and M –phase without intervening gap phases. The rapid nuclear divisions take place in a common compartment called a syncytium. An overview of early embryonic development in *Drosophila* as defined by Bownes stages (1975) is provided in figure 8 (143). Between Bownes stage 1-6, 13 rapid nuclear division cycles will have taken place along with 1 additional round of replication at the end to fill any gaps resulting from removal of damaged nuclei. Moreover, 6000 nuclei will be generated in the span of 3 hours at 25°C.

After the 8th nuclear division, which corresponds to Bownes stage 3, the nuclei begin to move out from the center to the surface. Some nuclei remain and these are called yolk nuclei. The yolk nuclei are polyploid and their function has not yet been elucidated. At Bownes stage 4 cellularization of the surface nuclei is initiated, whereby the developing embryo is now called a syncytial blastoderm. In a light microscope, cellularization can be visualized as a membrane begins to form around each surface nucleus. This is more apparent in Bownes stage 5 when the nuclei also acquire a more elongated than spherical shape (Fig. 8). Notably, cellularization is not completed until Bownes stage 6, which is when the developing embryo is called a cellular blastoderm (143).

During Bownes stage 4 and 5, which correspond to nuclear division cycles 10-13 and before surface nuclear cellularization completes, damaged nuclei sink and join the yolk nuclei in a Chk2-mediated process called nuclear fallout (144, 145). The damaged nuclei are hence not incorporated into the soma and are instead degraded alongside the yolk nuclei later in development. The resulting gaps from nuclear fallout are filled by new nuclei, which are generated from an additional round of cell division among neighboring nuclei. At Bownes stage 6 the surface nuclei fold inward (invaginate), which marks the start of gastrulation.

In Paper I, we observed that embryos deficient for Smc5 or Smc6 arrested in primarily Bownes stage 2 and 5, whereby the underlying reasons for these early developmental arrests were investigated.

3.2 BUDDING YEAST

Budding yeast, *S. cerevisiae*, is a unicellular eukaryotic organism that can propagate as either a haploid or diploid cell. When budding yeast are cultivated they initially grow as a sphere, but will eventually develop a bud that will form the daughter cell upon cell division. This gave rise to their given name as budding yeast.

As a model organism budding yeast are easy to handle, have relatively low maintenance cost and a short generation time. Moreover, many biological processes found in yeast are conserved in humans, which was more directly demonstrated in a study where Kachroo and colleagues (2015) systematically exchanged 414 yeast genes for their human orthologs (146). The authors showed that approximately 40% of the yeast genes could be replaced with their human ortholog.

3.2.1 Budding yeast mating

The genes involved in mating are primarily active during the cell cycle phase G1, whereby haploid yeast cells can respond to pheromones. By limiting mating to G1 the ploidy is maintained as both participating haploid yeast cells will have undergone cell division and are in the haploid state.

As haploids, budding yeast have a mating type of either MAT **a** or α, which is analogous to male and female gender. In *MAT*α cells, the gene product of *MAT*α1 and *MAT*α2 are expressed. *MAT*α2 forms a repressor together with a constitutively expressed protein called Mcm1, which inhibits transcription of **a**-specific genes (147, 148). Mcm1 also operates together with *MAT*α1 to activate expression of a group of α–specific genes, which include α–factor and Ste3 (149, 150). The former is a pheromone released by *MAT*α cells in order to attract *MAT***a** cells in the vicinity, whereas the latter is a *trans*-membrane receptor that binds **a**-factor released by *MAT***a** cells and enables *MAT*α cells to respond to *MAT***a** cells in the vicinity.

In contrast, *MAT***a** cells express *MAT***a1** and *MAT***a2** instead of the *MAT*α genes, whereby the **a**-specific gene products include Ste2 receptor and **a**-factor instead of Ste3 receptor and α-factor, respectively. In absence of *MAT*α1 no α–specific genes are transcribed and without *MAT*α2 the **a**-specific genes are not repressed.

Yeast cells of mating type **a**- or α produce a pheromone gradient when they release **a**- or α -factor, respectively. Yeast cells of opposite mating type can respond to each others pheromones by forming protrusions called shmoos. Mating initiates upon physical contact of two cells with opposite mating type. When haploid *MAT* -**a** and -α cells mate they form diploid *MAT* **a**/α cells, which do not undergo further mating. This is achieved by the expression of *MAT***a1** and *MAT*α2, which form an a1-α2 repressor that inhibits transcription of haploid-specific genes (151). Under nutrient poor conditions these diploids can, however, undergo sporulation. This is a meiotic process that generates four genetically distinct haploid cells. These haploid cells will re-enter the mitotic cell cycle and are able to mate again with their opposite mating-type partner.

3.2.2 Heterochromatin and its role in establishing mating-type in *S. cerevisiae*

In *S. cerevisiae*, a silenced copy of the mating-type genes α and **a** are present on chromosome 3 at loci called *HML* and *HMR*, respectively. Together with the transcriptionally active mating-type (*MAT*) locus, which also is present on chromosome 3, these loci regulate the mating type of a haploid yeast cell. The mating-type is set by the presence of either **a** or α information at the *MAT* locus, whereby *MAT***a** can be replaced by *MAT*α and vice versa by a so-called mating-type switch (Fig. 9).

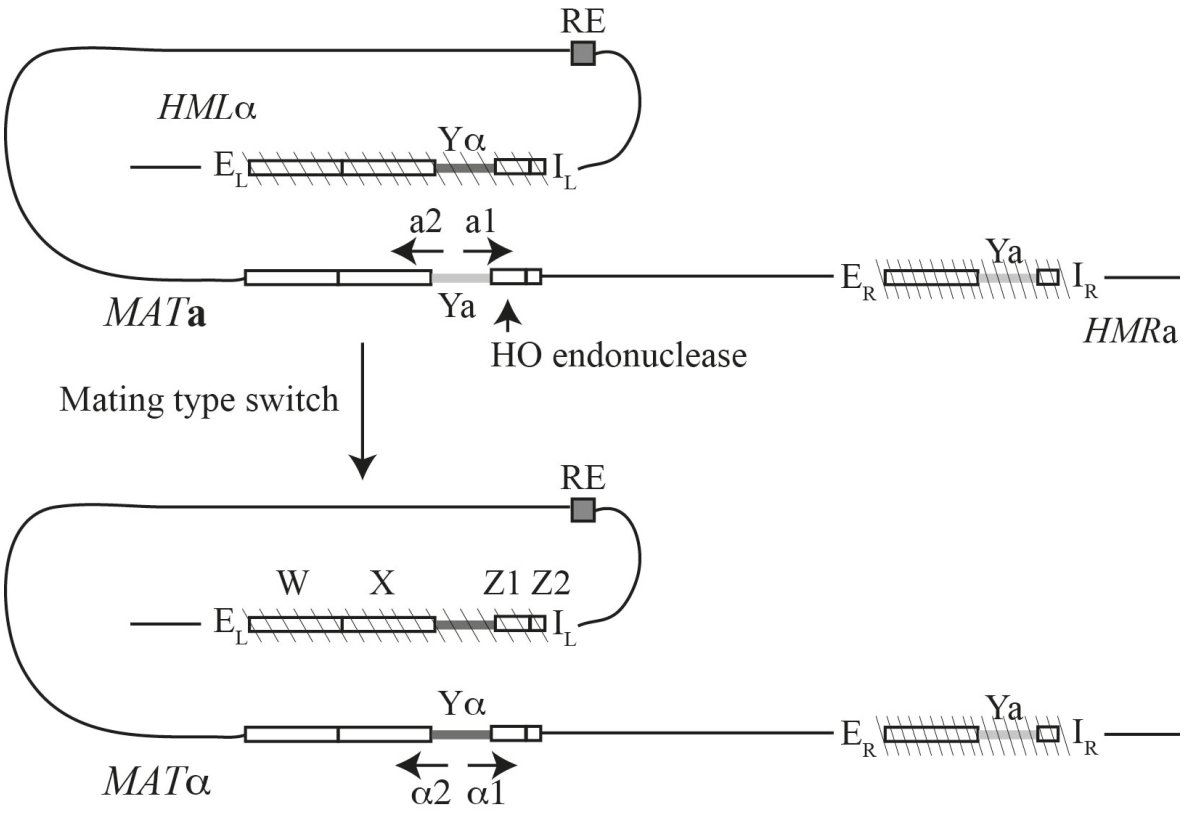

Figure 9. An overview of chromosome 3 mating-type loci and the mating-type switch. In this scenario, chromosome III of a *MAT***a** strain is undergoing a mating-type switch after HO endonuclease has introduced a cut as specified by an arrow downstream of *MAT*a1. The DSB is then repaired by using the HML cassette as a template through a recombination event called gene conversion, whereby the *MAT* locus then possesses a copy of *HML*α genes. RE is a recombination enhancer sequence, which influences template choice during mating-type switch. Both *HMR* and *HML* are flanked by an E and I silencer sequence. The diagonal lines, which cover both *HMR* and *HML* represent silencing. *HMR, HML*, and *MAT* share two regions, depicted as X and Z1, that are flanking the Y sequences. *HML* and *MAT* also share the regions depicted as W and Z2.

This mechanism is based on a DSB induction by the endonuclease HO (**ho**mothallic switching endonuclease) and subsequent repair via homologous recombination between *MAT* and either the *HMR* or *HML* locus. Notably, the pathway of mating-type switch is often inactivated to prevent a spontaneous change of mating-type in haploid yeast strains generally used in research. Mating between *MAT***a** and *MAT*α haploids creates diploid *MAT***a**/α cells, in which the expression of both **a** and α prevents further mating (152). Thus silencing of *HML* and *HMR* through heterochromatin formation is necessary to prohibit simultaneous expression of **a** and α genes, and enable mating of the haploid yeast cells. This is established at sequences known as the E- (*essential*) and I- (*important)* silencers, which flank both *HML* and *HMR*. Residing within the silencers are multiple *cis*-acting elements. These are binding sites, which recruit heterochromatin-establishing proteins. The well-characterized *HMR*-E silencer, for example, consists of three functional elements, a Rap1- (**R**epressor/**a**ctivator **p**rotein) and a Abf1- (**A**RS-**b**inding **f**actor) binding site and an ARS consensus sequence, which is a binding site for the Origin recognition complex (Orc) (153).

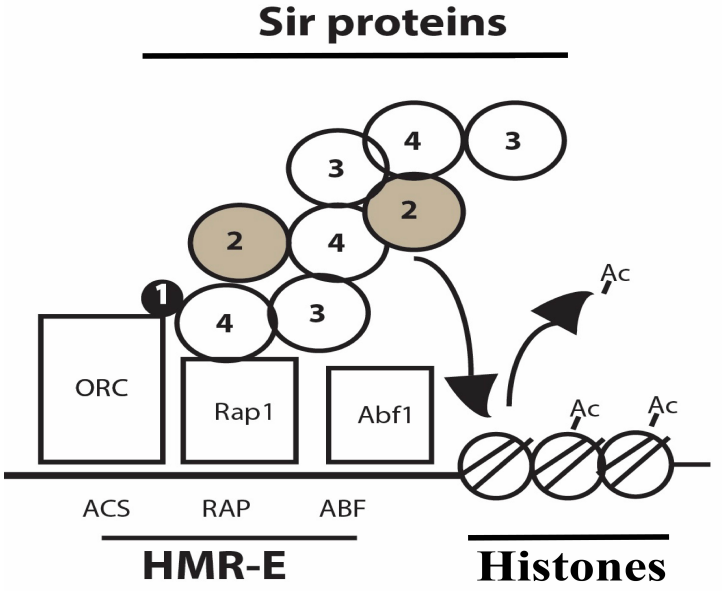

Figure 10. Overview of the *HMR*-E silencer and heterochromatin establishment. Sir proteins 1-4 are drawn as circles and denoted with numbers. Acetyl (Ac) group. The process of heterochromatin estabilishment is described in the main text.

In order to establish silencing at *HMR*, Orc1 recruits Sir1 (**S**ilencing **i**nformation **r**egulator 1) protein to the E-silencer (Fig. 10), which in turn promotes the recruitment and assembly of Sir proteins 2-4 (152). Sir2 is a NAD-dependent histone deacetylase, which deacetylates the nearby histone H3 and H4. Sir3 then binds to the deacetylated histones and recruits Sir4. Subsequently, Sir4 recruits an additional Sir2, which deacetylates the neighboring histone H3 and H4, and the process is repeated leading to establishment and spreading of heterochromatin. Heterochromatin formation is confined by so-called boundary elements, which act by establishing barriers that counteract heterochromatin spreading. The features of boundary elements include a lower affinity for Sir proteins, recruitment of acetyltransferases, and incorporation of histone variants such as H2A.Z (154).

4 METHODOLOGY

4.1 ANTIBODY GENERATION AND PURIFICATION

In response to foreign molecules in the blood stream, specialized plasma cells of the immune system known as B-lymphocytes secrete antibodies. Antibodies are glycoproteins that recognize foreign molecules. By exploiting the immune system of animals, researchers have been able to produce antibodies that recognize the presence of specific proteins of interest. They have taken advantage of the antibodies' ability to bind to their antigen (target) with high specificity and affinity.

In order to generate antibodies, an animal is injected with a polypeptide sequence of a protein of interest that will act as an antigen. Different B lymphocytes will bind the polypeptide at various points, which will be followed by a range of activating signals causing them to divide to produce memory B-cells as well as terminally differentiated antibody-secreting plasma cells (155). Each B-cell will generate an antibody that recognize a specific part of the polypeptide and since there was a mix of B cells there will be antibodies that recognize

various parts of the polypeptide. This population of antibodies is therefore called polyclonal. In contrast, monoclonal antibodies are a population of antibodies secreted from a single B cell and with a single specificity to the antigen. Köhler and Milstein pioneered the production of monoclonal antibodies, as they fused splenic B cells with myeloma cells resulting in hybridoma cells that divide endlessly. Each hybridoma cell can therefore be grown to produce antibodies with a unique specificity (156).

In paper I we used a polyclonal antibody that had been generated in guinea pig, which had been injected with a polypeptide based on the N-terminal amino acid sequence of Smc6. The serum was then extracted from the guinea pig at different time points and the antibody was purified from the sera.

For antibody purification the polypeptide used for immunization was expressed in bacterial cells together with an epitope-tag of histidine repeats via a galactose-inducible promoter. After incubation in media containing galactose the bacteria were lysed and the his-tagged polypeptide was extracted with the use of a nickel column. The histidine repeats bound more strongly to the nickel while other proteins flowed through or bound loosely. The purification continued by introducing increasing amounts of eluting solution containing imidazole, which is chemically reminiscent of histidine. At low amounts of imidazole, loosely bound unspecific proteins are washed off the nickel column as imidazole begins to compete for the binding of nickel. At higher amounts of imidazole the histidine repeats will be outcompeted and eluted. By collecting several fractions it was possible to pool the fractions that contained the highest concentration of the polypeptide. The polypeptide was incubated with a new nickel column followed by incubation with the sera. The antibody in the sera bound to the polypeptide while other protein flowed through or bound loosely. The antibody was eluted by a series of salt washes and the fractions with the highest concentrations of the antibody were then collected for dialysis with PBS. The antibody was later tested via western blotting.

4.2 PROTEIN GEL ELECTROPHORESIS

Gel electrophoresis has been used to separate various macromolecules in an electric field such as protein, DNA, and RNA. However, this section will focus on the aspect of gel electrophoresis as a tool in protein separation, which was used in paper I to examine expression of Smc6 in embryos. In this procedure denatured proteins are loaded onto a gel, which possesses a chemically generated 3D mesh or matrix that the proteins will migrate through when an electric field is applied across the gel.

Proteins extracted from cells are denatured through boiling in a loading buffer containing: sodium dodecyl-sulfate (SDS) to denature the proteins while also adding negative charge through its binding to the protein chain, a reducing agent such as 2-mercaptoethanol to break disulfide bridges, glycerol to make the sample more dense such that it can sink and remain in the wells of a gel, and a tracking dye to follow the migration of proteins.

In the denatured and reduced state the proteins are present in the form of a polypeptide chain. SDS anions bind to the amino acids in the polypeptide chain at about a ratio of one SDS anion per two amino acid residues. As such the SDS forms a complex with denatured protein with a large negative charge that is approximately proportional to the mass of the protein. This negative charge compensates the native charge of the protein. The proteins can be separated by mass when an electric field is applied across the gel. The mass of the proteins roughly correspond to their size, whereby larger proteins will become more hindered than smaller proteins in the 3D mesh of the gel (Fig. 11).

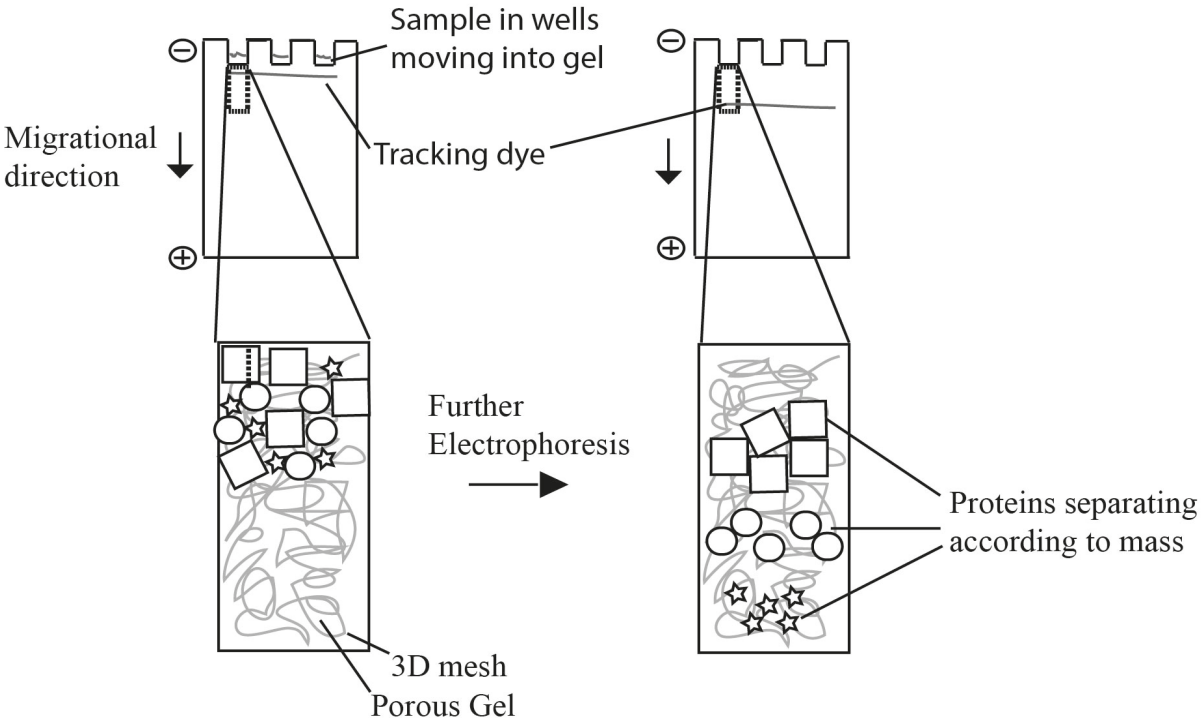

Figure 11. Demonstrating that different sized proteins are separated according to their mass when the electric field is applied during electrophoresis. The proteins are loaded into the wells of the gel. As an electric field is applied the sample enters the gel. The mixture of proteins move through a 3D mesh of the gel, but will not be noticeably separated according to their mass until later. Large proteins are more hindered and traverse less distance than smaller proteins.

4.3 CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) is a technique used to determine binding of a protein to a specific sequence in the genome (157). Cells are treated with a cross-linking agent, which generates a linkage of the proteins with DNA at close proximities. This means that proteins that are interacting with the DNA at the moment of cross-linking will remain linked there. The cells are then lysed. The DNA is subsequently sheared into smaller fragments with proteins still bound via techniques such as sonication or micrococcal nuclease digestion. Through the usage of specific antibodies coupled to beads the protein of interest along with the linked DNA can be extracted. The protein of interest in the sample is later degraded by proteinase treatment. This releases its bound DNA fragments, which can then be purified by

phenol chloroform extraction and DNA binding columns. The DNA fragments are then analyzed through; microarray, sequencing, or qPCR.

In paper II we performed ChIP by using an antibody that recognizes FLAG epitopes in order to isolate Smc6 from yeast cells expressing C-terminal FLAG-tagged Smc6 (Smc6-FLAG). qPCR analysis was subsequently performed to quantify the binding of Smc6-FLAG to a transcriptionally repressed region in budding yeast called *HMR*.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Early development of *Drosophila* embryos requires Smc5/6 function during oogenesis.

Aim and background: Studies into early development when Smc5/6 function is absent has been constrained by developmental arrest and death among mammals. Not surprisingly, very few patient cases have been identified and reported (1, 2). Fruit flies and their cells were recently shown to be viable in absence of a functional Smc5/6 complex (89, 90). Li *et al.* (2013) briefly examined embryos at the end of their development and claimed to observe no significant phenotype (90). No reports of Smc5/6 function at earlier developmental stages in *Drosophila* are available. We hypothesized that defects caused by nonfunctional Smc5/6 might be encountered and handled earlier in *Drosophila* development. Thus we aimed to investigate the function of the Smc5/6 complex during early embryo development in order to observe how *Drosophila* handle defects arising from dysfunctional Smc5/6, which might provide a clue as to why the complex is not essential.

Summary: Smc6-deficient flies were generated via P-element excision, whereby we used RT-PCR and western blot analysis to confirm the absence of Smc6 expression. In line with previous findings, the generated *smc6* mutant (*smc6*Δ^{35}) exhibited sensitivity when exposed to radiation or genotoxic agents. Another study of the Smc5/6 complex by Li *et al.* 2013, which focused on the later stages of *Drosophila* development, had generated an Smc5-deficient fly stock (*smc5*^{P7E8}) (90). Their *smc5* mutant strain was acquired and used alongside with our generated *smc6* mutant. Embryonic viability was quantified in both strains, whereby *smc5* and *smc6* mutants showed a significant reduction in embryonic viability in comparison to wild type flies. The embryonic phenotype of the *smc6* mutant flies was rescued through expression of an Smc6 transgene. Therefore, we concluded that the reduced embryonic viability was due to the absence of either Smc5 or Smc6 and that the Smc5/6 complex had an important function in embryonic development. Moreover, embryos derived from *smc6* mutant females mated to wild-type males exhibited a reduction in viability, whereas embryos derived from wild-type females mated to *smc6* mutant males showed normal embryonic viability. This indicated that Smc6 was a maternally contributed protein and as such it was possible that problems during embryonic development could originate already during oocyte (egg) development of *smc6* female mutants.

We explored both embryonic- and oocyte -development with the use of confocal microscopy. By collecting Smc5- and Smc6- deficient embryos and subjecting them to fixation at different time points we observed an accumulation of embryos halting at an early and late developmental phase. These two separated groups of embryos had distinct problems. In comparison to wild type, the early halting embryos had an altered nuclear morphology and the late halting embryos had nuclear material spread all across the syncytium. In combination with live cell imaging and embryos expressing a fluorescent protein linked to histone H2AV we observed that anaphase-bridged nuclei were being removed in a process called nuclear fallout. In nuclear stained embryos, we determined that embryos deficient of Smc5 or Smc6 had markedly more anaphase bridges formed among the late halting embryos. As such the observed delay at the late developmental stage could be attributed to the longer time needed for the embryo to remove anaphase-bridged nuclei from the surface by nuclear fallout.

Embryos that halted early, whereby the zygotic nuclei only underwent very few division cycles, have previously been associated with problems originating already in oogenesis. It was possible to confirm this by performing a scheme where the mother or the embryos were cultivated at either 18°C or 25°C. This scheme revealed a temperature-shift phenomenon, whereby improvements to embryonic viability were only present when the mother and thereby her oogenesis were cultivated at 18°C. Mothers cultivated at 25°C, laid eggs containing embryos with no improvement in viability even if the embryos were allowed to complete their development at 18°C. This highlighted that embryonic viability in absence of Smc5/6 relies on events occurring during female meiosis and oogenesis. In a nondisjunction (NDJ) assay, Smc6-deficient flies were observed to have an increased NDJ frequency compared to wild type, but the crossover rate appeared to be unaffected and the increased NDJ was much lower than for canonical nondisjunction mutants. Intriguingly, the NDJ observed in Smc6-deficient embryos was largely confined to the missegregation of the *X*-chromosome, which also is the sole chromosome with an rDNA locus. An examination of oocytes from Smc5- or Smc6- deficient females revealed several abnormalities including persistent DNA DSBs, delay in oocyte selection (pachytene arrest), and an altered karyosome morphology and dynamics. Surprisingly, no defects in either the patterning of the eggs or the ventral-dorsal axis were found among Smc5- or Smc6 –deficient eggs, which was further confirmed by proper Gurken expression and localization.

Ultimately, all defects found in embryogenesis and oogenesis of *smc5* and *smc6* mutants were resolved by either cultivating the mother (and her oocytes) at a lowered temperature or in the presence of an Smc6 transgene. We therefore concluded that the Smc5/6 complex has an important role in genome integrity and replication completion during oocyte- and embryonic –development. Moreover, data from the temperature experiments indicate that problems that arise during oocyte development in *smc6* and *smc5* mutants could be passed onto developing embryos, which do not recover even if allowed to developed at a lower cultivation temperature.

Perspective: This was the first time that the Smc5/6 complex had been studied in early *Drosophila* development, whereby events from oogenesis to early embryogenesis were accounted for. Moreover, we discovered that embryos lacking Smc5 or Smc6 suffered from developmental arrests and decreased embryonic viability as a result of genomic instability. This was contrary to the report by Li *et al.* (2013), which claimed to observe no significant phenotype among embryos (90). Our findings demonstrate that early development in *Drosophila* can be an attractive platform to further investigate Smc5/6 function.

Intriguingly, a temperature-shift phenomenon pinpointed that an early halt in embryogenesis might stem from problems occurring during meiosis in the absence of Smc5/6. Temperature affects many things, but it is not the first time that temperature has been demonstrated to have an impact on meiosis. Plough (1917) described how temperature changed chromosomal form and behavior, whereby a temperature shift caused a change in internal coiling frequency and positioning of crossovers (158).

Surprisingly, the unrepaired DSBs found during oogenesis did not lead to development termination and apoptosis. This is, however, in line with a previous study that showed how a weak mutant allele of a DSB repair protein called *okr* evaded checkpoint arrest and apoptosis due to a low number of DSBs (62). This implied that the mei-41 DSB checkpoint has a threshold for the amount of DSBs required for its activation. In line with this, Gurken expression appeared normal in eggs lacking Smc6, whereby if the checkpoint activation would have disrupted expression and/or localization of Gurken. We therefore hypothesized that low numbers of DSBs are invisible for the checkpoint and thus are carried over into embryogenesis along with other defects such as altered karyosome morphology and dynamics. Interestingly, while a subset of embryos halt early on in development as a result of defects in oogenesis, many embryos are still able to proceed. In line with this, *smc5/6* mutants have previously been shown to override a checkpoint arrest and proceed with the cell cycle, which resulted in mitotic catastrophe (159). Fortunately, *Drosophila* has a process called nuclear fallout, which removes damaged nuclei that undergo mitotic catastrophe during embryogenesis before they are incorporated into the soma. Inactivation of the nuclear fallout process in combination with an *smc6* mutant reduced embryo survival drastically (data not shown). Thus, *Drosophila* has developed an efficient survival mechanism that allows them to cope with extreme chromosomal abnormalities even in the absence of essential protein complexes such as Smc5/6.

This study did not examine the relationship with cohesin or condensin, but such a relationship would be interesting to evaluate in the future. Moreover, a method that enables researchers to study the rapid nuclear divisions *Ex vivo* was recently developed (160). The report demonstrated how an embryonic cytoplasm could be extracted, whereby the rapid nuclear divisions could proceed outside the embryo while being monitored under a fluorescence microscope. This technique can enable researchers to directly manipulate or supplement the extracted cytoplasm with a desired drug or chemical while monitoring any changes under a fluorescence microscope. For our purposes, such a technique would enable the use of *smc5/6*

mutants, which generate many anaphase bridges. Thereby resolution of anaphase-bridges could be studied more intimately with specific addition of gene products or chemicals.

5.2 PAPER II

Smc6 and Top1 prevent aberrant recombination at the silent mating-type locus *HMR* in budding yeast

Aim: The Smc5/6 complex had been shown to localize to transcriptionally repressed regions in both challenged and unchallenged conditions. As a result, Smc5/6 was implicated in maintenance of transcriptionally repressed regions through its function in DSB repair and recombination. Our own ChIP-on-chip results (own data, not shown) indicated that the Smc5/6 complex in budding yeast binds to the transcriptionally repressed region *HML*. This binding was peculiar as it was present throughout all cell cycle stages and independent of Top2 and Cohesin, which is in contrast to the known association of Smc5/6 to other genomic regions. We therefore hypothesized that Smc5/6 might have a novel function at *HML* and possibly its related, but distanced region *HMR*.

Summary: In paper II, we initially investigated if Smc5/6 had a function in heterochromatin maintenance and boundary formation at *HML*. This was done using cells with a temperature sensitive *smc6-56* mutant allele and a *URA3* reporter gene inserted at the *HML* locus, or at either 1 or 4 kb downstream of the *HML*-I silencer. Expression of *URA3* allows cells to grow in the absence of uracil in the media, and renders cells hypersensitive to 5-Fluororotic Acid (5-FoA). By plating serial dilutions of cells on agarose plates with or without uracil and with or without 5-FoA at permissive temperature, we concluded that Smc5/6 did not have any apparent function at heterochromatin maintenance or boundary formation at *HML*. In line with this, *HML*α2 expression in MAT**a** cells was measured by northern blot analysis, whereby *smc6-56* cells grown at non-permissive temperature showed robust transcriptional repression of α2 similar to wild type cells. Next we examined if Smc6 was able to bind to *HMR* by using ChIP-qPCR. In wild type cells Smc6 binds to *HMR*, which was abolished in cells lacking Sir2. Thus, we concluded that Smc6 binding to *HMR* occurs in a heterochromatin-dependent manner.

We next explored if Smc6 binding to HMR reflected a novel function in heterochromatin establishment. To address this we used a mutant strain that carried a crippled *HMR*-E silencer with a disrupted binding site for Rap1 and Abf1 (153, 161). The combination of the two disrupted binding sites were denoted as *HMRae*. Notably, MATα *HMRae* cells mate inefficiently due to the simultaneous expression of **a** and α information from the *MAT*- and *HMR* –locus, respectively. A mating assay previously used to indicate silencing at *HMR* revealed that MATα *HMRae smc6-56* cells could improve mating efficiency as compared to MATα *HMRae*. This was reflected by the larger subpopulation of MATα *HMRae smc6-56* cells that formed diploids as compared to wild type when introduced to a MAT**a** mater strain.

Moreover, in line with the ChIP-qPCR data, *smc6-56* could not improve mating efficiency in absence of Sir2.

Intriguingly, the ability of *HMRae smc6-56* cells to increase mating efficiency was dependent on Rad52, which is required for HR in budding yeast. Deleting *RAD52* abolished mating efficiency of MATα *HMRae smc6-56* cells. We therefore postulated that the increased mating efficiency was mediated through an aberrant recombination event during DNA replication in *smc6-56*. Rad52 is involved in restarting and repairing replication forks that have either stalled or collapsed, respectively. To test our hypothesis that abnormal replication progression could initiate aberrant recombination at *HMR* and subsequently increase mating efficiency we tested mating efficiency in a *top1* mutant. Previously *top1* and *smc6* was shown to share a phenotype of replication delay for long chromosomes, which implied that replication fork progression might be impaired when either protein was functionally absent. We therefore tested MATα *HMRae top1Δ* cells in the mating assay, which revealed a similar ability to improve mating efficiency as MATα *HMRae smc6-56* cells. Moreover, the ability of MATα *HMRae top1Δ* cells to improve mating efficiency also depended on Rad52. Conversely, deletion of Rad52 did not affect the ability to improve the mating efficiency for an acetyltransferase mutant (MATα *HMRae* rtt109Δ), which was previously shown to restore silencing through lack of acetylation of histone H3 (161). Altogether, this indicated that MATα *HMRae smc6-56* cells most likely encounter problems during the replication of *HMR*. Moreover, data from the mating assays revealed that MATα *HMRae smc6-56 top1Δ* cells restored mating efficiency to a level similar to MATα *HMRae smc6-56.* Thus, Top1 and Smc6 might operate together to ensure proper replication progression at *HMR.*

As both MATα *HMRae smc6-56* and MATα *HMRae top1Δ* cells were dependent on Rad52 to improve mating efficiency, we wondered if the HMR region had been altered in the subpopulation of cells that had managed to mate and form diploids. To investigate this we collected diploids from the mating assay and examined the mating products by PCR, which indicated that half of the mating products could no longer produce an amplicon of either *HMR*-E or *HMRA1*. This indicated that cells of either MATα *HMRae smc6-56* or MATα *HMRae top1Δ,* which had managed to mate, corresponded to cells that had undergone alteration or re-arrangement of the *HMR* region depended on Rad52. Further molecular biology experiments are, however, needed to evaluate and characterize this re-arrangement. Ultimately, this demonstrated that cells of either MATα *HMRae smc6-56* or MATα *HMRae top1Δ* improved mating efficiency via genomic alteration of *HMR* as opposed to conventional transcriptional repression. Although, this study does not exclude that Smc6 or Top1 might still have role in transcriptional repression.

Perspective:

We observed that Smc6 bound to the heterochromatic locus *HMR* on chromosome 3 in budding yeast. ChIP-qPCR revealed that Smc6 binding to *HMR* was heterochromatin-dependent, since binding was abolished in a *sir2* mutant. In line with this, heterochromatin-

dependent binding of Smc6 has been demonstrated in other organisms (120, 123). Notably, this study could not identify a heterochromatin-specific role for Smc6 at either *HML* or *HMR*. However, such a role was not excluded, whereby Sir2-dependent binding of Smc6 to *HMR* remains an interesting observation. Moradi-Fard and colleagues (2016) recently demonstrated that Nse3 and Smc6 interacted with Sir4, which mediated Sir4 localization to the telomeres. Absence of Nse3 resulted in loss of transcriptional repression at the telomeres along with telomere shortening defects (125). Recently, Smc5/6 was also implicated in transcriptional repression of episomal genes of the Hepatitis B virus (126, 128). Thus, the exploration of Smc6 function in transcriptional repression and at transcriptionally repressed regions are of high interest.

An intriguing aspect of our study was that Top1 had not previously been associated with modulation of recombination at any loci other than at rDNA. Rad52 was required for MATα *HMRae top1Δ* cells to improve mating efficiency, which suggest that Top1 function also extends to the stability of *HMR*. In contrast to *top1Δ*, *smc6* mutants have previously been linked with aberrant recombination and genomic rearrangements (94, 107, 122). Furthermore, *smc6* mutants have been shown to accumulate x-shaped structures at stalled and collapsed forks (55, 98). As MATα *HMRae smc6-56* and MATα *HMRae top1Δ* cells share a similar Rad52-dependent phenotype and because the MATα *HMRae smc6-56 top1Δ* cells has similar phenotype as MATα *HMRae smc6-56* cells, we propose that Top1 and Smc6 operate together to ensure proper replication progression. In line with this notion, Smc6 and Top1 have previously been implicated in promoting proper replication fork progression on longer chromosomes (113). We also propose that in absence of either functional Smc6 or Top1 replication fork progression becomes compromised, whereby aberrant recombination takes place via Rad52.

To confirm our hypothesis further additional experiments are required. For example, to identify the recombination events that had occurred at *HMR* in *smc6* and *top1* mutants DNA sequencing and DNA southern blotting needs to be performed to help clarify the role of Smc5/6 at heterochromatin.

6 CONCLUDING REMARKS

In absence of Smc5/6 cells exhibit problems in DNA replication, DSB repair, and chromosome segregation. These problems cause genome instability, which if tolerated culminate in developmental disorders and disease (1, 2, 162). Moreover, Smc5/6 was recently implicated as a host viral restriction factor against the Hepatitis B virus (126, 128) and associated with brain metastases (3). Altogether, these observations highlight the scientific importance and medical relevance of understanding Smc5/6 function. We have studied Smc5/6 function in unchallenged conditions in an attempt to gain further insight into the function(s) of Smc5/6. Furthermore, we wanted to understand why Smc5/6 was essential in some organisms, such as yeast, compared to the tolerance of absence of Smc5/6 in others like *Drosophila*.

Smc5/6 is not essential in *Drosophila*, whereby Li *et al.* (2013) claimed to observe no significant phenotypes among embryos lacking Smc5/6 (90). We have demonstrated the contrary by showing that embryos deficient of Smc5/6 halt in development due to genome instability, which manifest as DSB repair and chromosome segregation problems. Moreover, our data indicate that defects during female can be propagated throughout egg development and perturb future embryonic development. Despite all these defects, a significant amount of *Drosophila* embryos were still viable and able to proliferate. This could largely, but not exclusively, be attributed to two features in *Drosophila*: higher DNA DSB tolerance during oogenesis enabling oocytes to circumvent apoptosis and an ability to remove as well as replace damaged nuclei during embryogenesis to evade termination and death during development. Altogether, our investigation has shown that early *Drosophila* development is an attractive platform to further study Smc5/6 function in unchallenged conditions. In budding yeast, we showed that Smc5/6 binds to heterochromatin, but we could not identify any apparent heterochromatin-specific function. Instead our data indicated that Smc5/6 prevents aberrant recombination. Intriguingly, absence of Top1 revealed a similar phenotype as seen when Smc5/6 is mutated. As Top1 resolves superhelical tension, this may indicate that regulation of superhelical tension is vital to prevent aberrant recombination and that Smc5/6 works alongside Top1 to resolve replication-induced superhelical tension.

The aim of our research was to understand Smc5/6 in DNA DSB repair, replication, and chromosome segregation during unchallenged conditions. We tried to address these functions in conjunction with *Drosophila* embryo development and yeast heterochromatin biology and thereby provided important insights into the complex role of Smc5/6 in genome maintenance.

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